DEATH OF THE CORTEX OF ROOTS IN RELATION TO INVASION BY FUNGAL PARASITES

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ABSTRACT

In glasshouse and laboratory conditions, death of root cortices of cereal and tomato plants was followed by nuclear and cytoplasmic staining methods. Various treatments were applied to whole plants or sterile root pieces on agar, to study factors that influence root tissue senescence and its relationship to invasion by the parasitic fungi *Microdochium bolleyi*, *Pyrenochaeta lycopersici* and *Gaeumannomyces graminis* var. *tritici* (*Ggt*). Other experiments involved growth and sporulation of *M. bolleyi* in culture, and the ability of this fungus to spread on roots by spores produced on seeds or roots, to facilitate development of *M. bolleyi* as a seed-applied biocontrol agent of plant pathogens. Nuclear staining with acridine orange was satisfactory for assessing root cell viability in cereals but not in tomato, for which neutral red/plasmolysis was used; no other cytochemical method was suitable for routine work with either plant. Sterile detached root pieces of wheat and tomato senesced rapidly on media without sucrose, but tomato root cells remained alive on media of low sucrose concentration (0.1%), whereas wheat root cortical death (RCD) was delayed but not inhibited by even high sucrose levels. RCD was also delayed by removal of the shoot, root tip or seed from sterile wheat seedlings; supply of sucrose or minerals to only part of a root piece did not affect the pattern of RCD. Indolylacetic acid, Ag^{+} or Co^{2+} delayed RCD in wheat root pieces on agar, but other growth regulators accelerated RCD. The results suggest that RCD in cereals is programmed and internally regulated, being only slightly modified by external factors.

M. bolleyi invaded senescing cereal root cortices mainly intercellularly. It accelerated RCD of detached roots but had little effect on attached roots; it invaded tomato roots only poorly.

P. lycopersici invaded cereal and tomato roots mainly intracellularly and killed the tissues, indicative of a different growth habit. Wheat, barley, oats and rye showed different rates of RCD in root pieces on agar, and oats had a different pattern of RCD from that in other cereals. *Ggt* killed roots of all four cereals, attached and detached from seedlings, but it penetrated poorly into oat roots even when their cells died. An attempt was made to relate these events to patterns of induced lignification in root cells.

The rate of RCD of glasshouse-grown wheat could be manipulated by nitrogen or calcium supply, with effects on colonisation by *M. bolleyi*. Pruning of leaves and urea foliar sprays did not affect RCD nor colonisation by *M. bolleyi* or invasion by *Ggt*. Roots of glasshouse-grown tomato did not show RCD equivalent to that in cereals; root cortical cells remained alive even after removal of the shoot. There was evidence of death of small feeder roots, but experiments to relate this to invasion by *P. lycopersici* were inconclusive.

M. bolleyi sporulated abundantly when applied to wheat seeds in tubes or cups of perlite, and the spores could be assayed in water draining from the containers. Removal of the seeds and direct plating of roots at different times showed that the fungus also sporulated on roots, colonising their senescing cortices.

In culture *M. bolleyi* required only glucose, mineral salts and thiamine for growth and sporulation. By videomicroscopy some germinating spores were seen to undergo microcycle conidiation, which was suppressed by nutrients and in the vicinity of root pieces. These and other results are discussed in relation to the roles of weak parasites in colonising naturally senescing root tissues.

TABLE OF CONTENTS

SECTION 3: ASSESSMENT OF ROOT CORTICAL DEATH IN STERILE ROOTS ATTACHED TO OR DETACHED FROM SEEDLINGS AND ITS IMPLICATION FOR PARASITIC FUNGI

1. Introduction

It has long been known that the physiological condition of the plant host plays a major role in the development of host-parasite associations. There are, for example, many stress-induced diseases in which fungi that are normally considered as only weakly virulent, can invade plants with diminished resistance due to injurious environmental conditions. The fungi that cause the stalk rot/root rot syndrome of maize are classic examples of this (Dodd, 1980). Other fungi, such as *Colletotrichum* spp. that cause fruit spots, exploit the natural senescence of host tissues. Their spores germinate to form pigmented appressoria that persist on the fruit surface until the fruit begins to ripen, when they invade the host tissues (Binyamini & Schiffman-Nadel, 1972). Yet other fungi, such as *Botrytis cinerea*, characteristically invade healthy plant tissues from a food base provided by senescent tissues such as the dead and dying remains of flowers. *B. squamosa*, a foliar parasite of onions, infects and grows faster on the older senescing leaves than on young healthy leaves (Alderman & Lacy, 1984).

Garrett (1970) described such behaviour as characteristic of unspecialised parasites, whereas specialised parasites can invade plants of full vigour. Yarwood (1976) also discussed this subject, defining vigour as the opposite of senescence, and he noted that facultative saprophytes are commonly favoured by senescing tissues whereas some fungi, notably the obligate parasites such as powdery mildews and rusts, are favoured by host vigour. These extremes of behaviour are undoubtedly true. However, research on cereal roots, discussed later, has indicated that even specialised necrotrophic parasites can benefit from senescence of host tissues. Senescence

thus has an important role in the understanding of host-parasite interactions - the subject of this thesis.

1.1. Senescence and ageing of plant tissues

Plants physiologists have intensively studied senescence processes, and the subject has been extensively reviewed (Thimann, 1980a; Nooden & Leopold, 1988). A distinction is commonly made between senescence and ageing. Cell senescence can be defined as the sequence of changes occurring with time and which results in the decreased survival capacity of the cell. Senescence represents endogenously controlled degenerative processes leading to death while ageing encompasses a wider array of passive non-regulated, degenerative processes driven primarily by exogenous factors and which occurs as a late developmental process. (Medawar, 1957; Leopold, 1975; Woolhouse, 1980; Gahan, 1982). Further ageing covers a wide span of physiological changes, some of which may lead to weakening of the organism and decrease in its resistance to stress, so increasing the probability of death, while other changes may have little or no effect on the capability of the organism to survive.

Many of the authors above recognise that it is characteristic of plant development that senescence is not simply a running down of the life process but is a highly ordered and programmed process or series of processes. Many of the manifestations of senescence are facets of development carried out in an orderly fashion and serving a wide variety of adaptive functions. The idea that death might be actively induced by endogenous factors that are a natural part of the development of the organism has thus been gaining support. In other words, programmed cell death may be regarded as a mechanism for survival rather than destruction.

Most of the work on plant senescence has involved above-ground organs such as leaves, flowers and fruits, or it has involved whole plants such as in annuals that have a limited natural life span. Much less information exists for root senescence.

Plants, according to their habit of growth, senesce in many different ways. The whole plant may senesce and die at one time, as occurs in annuals or biennials after flowering and setting of seeds. Molish (1929, transl. 1938) in his classical book considered that this kind of senescence is due to the migration of major amounts of organic constituents from the leaves into the fruits, i.e. senescence is caused by starvation. Alternatively, there may be a progressive senescence of plant organs such as leaves and flowers. Finally, during the process of tissue maturation certain cells, such as xylem vessels and tracheids or sclerenchyma tissue, may senesce and die although the plant as a whole is in a state of vigorous growth (Ġahan, 1982).

Patterns of senescence appear to differ in quite important ways in terms of their causes, the nature of the senescence process or the degree of reversibility. Some types of senescence appear to be closely correlated with developmental events in the whole plant. For example, senescence of monocarpic plants is closely related to the process of flowering and the growth of fruits. If flowers or fruits are removed then senescence may be postponed. Thus the senescence that follows fruit production or the senescence of flower parts after fertilisation is apparently irreversible and is an inevitable consequence of flowering or fruiting. Once started, it cannot be influenced by internal or external stimuli. On the other hand, the rapid senescence of a detached leaf can be reversed and the leaf rejuvenated by the application of cytokinins or by allowing the leaf

to root. Similarly, older leaves of plants like bean or tobacco senesce as the plant grows. This senescence can be reversed if the top of the plant is cut off. These observations suggest that there may be more than one process and causal agent of senescence and cell death, characteristic of different situations and different tissues.

The term senescence has sometimes been restricted to those processes that follow the "point of no return" (Wang & Woolhouse, 1982). Yet the changes that occur before the point of no return do not seem less important than those that take place afterwards (Nooden & Leopold, 1978) and they may well be the transforming processes that drive the senescence syndrome. There are numerous reports of reversal of senescence at fairly late stages (reviewed by Nooden, 1988a) so senescence need not be irreversible, although its end point, death, is so.

The various parts of the plant influence each other in ways that serve to achieve a coordination of developmental processes that involve senescence (Sutcliffe, 1976; Moorby, 1977; Wareing, 1977; Goodwin *et al.*, 1978). The oldest, and so far most successful, approach to analysing these correlative controls has been by surgery; for example, the abscission of one structure to permit analysis of its effects on another. The earliest and most extensive literature on such correlative controls concerns flowers (reviewed by Nooden 1988a). Nevertheless, the complexity of such whole plant systems makes them difficult to analyse. For this reason it is attractive to study simpler systems such as detached plant organs. This has been done extensively with detached leaves (reviews by Thimann, 1980b; Thomas & Stoddart, 1980), flowers (Rogers, 1973) and fruits (Rhodes, 1980). The problem is that excision may alter the process of senescence and the responses to hormones; it may also

cause ultrastructural changes and it blocks the movement and distribution of nutrients (reviewed by Nooden, 1988a). Therefore excised organs are valuable only insofar as the senescence processes in them can be related to events in intact plants.

1.1.1. Factors affecting senescence

Natural senescence is genetically programmed and each species, individual and tissue has its own pattern of senescence. However, environmental factors may affect this pattern. Conditions which induce stress in plants, such as extreme temperatures, reduced illumination, drought, lack of oxygen and organic or mineral nutrient deficiencies, usually enhance senescence. Finally, exogenously applied growth regulators affect senescence. Indeed, hormones are generally considered to be implicated in natural senescence processes (Wareing, 1977; Goodwin *et al.*, 1978; Leopold & Nooden, 1984). The senescence-promoting effects of ethylene and the delaying effects of cytokinins are well-known examples of this level of control. The genetic regulation of senescence and the ways in which this is modified by environmental factors are only now beginning to be studied. It is believed that programmed senescence may involve differential gene activity, differential synthetic activity in terms of enzymes and proteins and also differential cell lysis or destruction. However, there is no certain pattern of metabolic change that has been found to be characteristically associated with senescence. Farcas (1978) summarises the metabolic changes occasionally observed in senescing plant tissues as follows. 1. Decreased photosynthesis (in leaves). 2. Changes in respiration, usually involving a decreased rate of respiration and loss of control of mitochondrial function, although there may be an increase

in respiration at the start of the process. 3. Changes in nucleic acid turnover. 4. Changes in protein metabolism. 5. Abnormal protein production. 6. Changes in enzyme activities. 7. Changes in lysosomes, in that the amounts of lysosomal hydrolytic enzymes greatly increase during senescence. 8. Accumulation of secondary metabolites. 9. Changes in hormonal balance. 10. Increased cell permeability, suggesting damage of the cell membranes.

It is not known which, if any, of these features is central to the senescence process. However, cytochemical and ultrastructural studies have identified the end of the senescence process in which there is a loss of compartmentation in the cell which almost certainly is lethal. Senescing cells undergo changes of structure, and much of the membrane system is disrupted (Varner, 1961; Gahan, 1982). It has been suggested that the vacuole acts as a lysosome, secreting hydrolytic enzymes which digest cellular material that is no longer needed (Matile, 1975, 1978). However the situation is not so simple as this because the internal structure of chloroplasts and mitochondria is also reduced and this seems to happen before their external membranes are disrupted. Therefore it seems likely that degradative processes are initiated or synthetic processes eliminated in organelles as well as in cells. Possibly the same signal that causes senescence in cells is also perceived by their organelles, causing them to senesce simultaneously.

1.1.2. Assessment of senescence and death

From the practical viewpoint it is necessary to identify senescence or cell death by some simple criterion so that the process can be recorded and related to other events such as invasion by pathogens. But, given the uncertainty over what is central to

senescence, the choice of a single criterion is difficult. Depending on the needs of individual studies, senescence can be assessed by the loss of chlorophyll in leaves or the lowering of protein levels (Thimann, 1980b; Thomas & Stoddart, 1980). Leakiness of the cell membranes may also be a good parameter (Halevy & Mayak, 1979) although increases in leakage rate occur relatively late in the senescence process. An additional difficulty is that there is no definite boundary between the living and dead states of a cell, nor is there any single criterion that can be related to the point of no return. Techniques based on features such as plasmolytic responsiveness, exclusion of certain dyes, cytoplasmic cyclosis and measurements of a cell's reducing capacity through the use of redox dyes can give different assessments of cell senescence and death in different types of tissue. One example of such variability is that the breakdown of the nucleus is usually a late event in most senescing cells but it may occur relatively early in xylem cell differentiation (Lai & Srivastava, 1976; Gahan, 1982). Nevertheless, there are underlying similarities in the features of senescing cells, especially at the ultrastructural level (Butler & Simon, 1971; Halevy & Mayak, 1979; Gahan, 1981, 1982).

Some of these features relating to senescence in plants in general were examined in the context of root senescence in this thesis. They were considered important, not only because senescence of root tissues has been relatively little studied but also because root tissue senecsence may have a major role in the establishment of microbial populations on or in roots.

1.2. Senescence of roots

The senescence and shedding of root tissues has been known for a long time but only quite recently has it attracted the attention of plant physiologists or rhizosphere biologists, and still more recently of root pathologists. One of the main reasons for this is that root senescence was thought to occur relatively late in the life of roots or of plants as a whole. However, it is now thought that root tissue senescence occurs relatively early in the lives of plants - early enough to have major implications for microbial populations in the root zone and, through the activities of these microorganisms, major implications for plant health.

The concept of the rhizosphere, as first defined by Hiltner (1904), has been modified in recent years to take account of the senescence of root cortical tissues. Old & Nicolson (1975) were among the pioneers in this field when they recognised the existence of an endorhizosphere in sand-dune grasses because various fungi and bacteria were abundant in the root cortex of these plants. This paralleled the earlier work of Waid (1957, 1974), to be described later. More recently, Rovira *et al.* (1979) classified the sources of nutrients for microorganisms in the rhizosphere. The five categories of source of nutrients that they distinguished can be simplified to three, as follows.

(1). Exudates, comprising water-soluble materials such as sugars, amino acids, organic acids, hormones and vitamins which leak from the root without the involvement of metabolic energy.

(2). Secretions, including polymeric carbohydrates (mucilages) and enzymes which depend upon metabolic processes for their release, and coming mainly from the root cap zone but also from the extension

zone and the root hair zone. Some simple organic nutrients may also be released by metabolically dependent secretion.

(3). Lysates, released when cells autolyse. This includes release of materials from cell walls, from whole cells such as root hairs and from sloughed cortical and root cap cells. The root cap of *Zea mays*, for example, may slough more than 4000 cells per day (Clowes, 1969; Moore & McClelen, 1983). Eventually, of course, the whole root lyses.

The complexity of this whole topic is illustrated by the fact that Hale *et al.* (1982) tabulated forty factors that are known to affect root "exudation", although whether they do so by influencing exudation, secretion or lysis has often not been determined.

Numerous studies have shown that a relationship exists between the size and composition of the saprophytic flora on roots and the amount of the root degeneration. Waid (1957) found that there is a sequential change in the types of fungi on rye-grass roots with increasing degree of cortical degeneration. Dix (1964) made similar observations on bean roots.

Barber & Lynch (1977) calculated that the biomass of the microbial population on cereal roots was too high to be accounted for by the amounts of carbon shown to be released from roots of gnotobiotic plants, the implication being that the microbes must enhance the release of carbon nutrients. A similar suggestion has been made by Barber & Martin (1976) and Martin (1977), based on experimental work, although Newman (1985) drew attention to some discrepancies or interpretational problems in these studies and noted the evidence for microbially-enhanced release of carbon from roots is questionable. Whipps (1989) recently reviewed this topic. In any case, the amounts of carbon released by functional roots are

considerable. Martin (1977) found that of the carbon transported to wheat roots over 23 days at 10°C, an average of nearly 40% was lost to the soil, possibly largely from the breakdown of the cortical cells. Griffin *et al.* (1976) showed also that death and sloughing of root cortical cells of peanut plants can occur in gnotobiotic conditions and amount 0.15% of the total root organic matter per week.

Newman (1985) recently made a critical review of this whole topic in relation to the rhizosphere population. By analysing various published data, he concluded that soluble exudation is often in the range of 10-100 mg g^{-1} root and insoluble root material released from still functioning roots is often 100-250 mg g^{-1}.

In terms of the location and activities of rhizosphere organisms, scanning and transmission electron microscopy has shown that bacteria are often confined to the grooves between rhizodermal cells that contain normal cytoplasm and complete membranes (i.e. living cells), but are attached to the whole of the outer tangential cell surface of cells with disorganised cytoplasm and irregular plasmalemma and tonoplasts (i. e. moribund cells) (Foster & Bowen, 1982; Foster, 1986). These observations suggest that there is a change in the distribution of microorganisms at the rhizoplane as the cortical cells die. As the rhizodermal cells autolyse, their cell walls become impregnated with suberin (Esau, 1965), a complex mixture of polyphenolics, lipids and epoxides. Despite suberization, however, the rhizodermal cells soon become invaded by microorganisms. With the breakdown of their membranes the rhizodermal cells lose turgor and collapse, and both TEM and SEM studies show that the pressure of the surrounding soil may cause the outer tangential wall to become inverted so that the radial

longitudinal cell walls stick out like ribs into the rhizosphere, and soil minerals enter the space formerly occupied by the cell lumen. As the flow of lysates ceases, so rhizosphere bacteria are thought to attack the primary wall of the rhizodermis, removing the gel-rich layer (Foster, 1982; Foster *et al.* 1983). Invasion of the cortex establishes a microbial continuum from the outer rhizosphere almost to the stele (Old & Nicolson, 1975).

1.2.1. Root Cortical Death (RCD) in Gramineae

The death and sloughing of root cortices of the Gramineae has been documented repeatedly (Dunn, 1921; Weaver, 1926; Troughton, 1957; Garwood, 1967; Waid, 1974; Scott-Russell, 1977), but has been assessed mainly by the degree of root browning. This is an unreliable criterion as Kirk & Deacon (1987b) found in the case of cocksfoot (*Dactylis glomerata*) roots. Beckel (1956) seems to have been the first person to assess cortical senescence in grass roots by a reliable criterion. She used nuclear staining methods (Feulgen reagent) for the Canadian prairie grass, *Bouteloua gracilis*, and found that the cortical cells lost the ability to exhibit nuclear staining in root regions only a few days or weeks old. Moreover, Beckel's (1956) work demonstrated a clear pattern of cortical senescence, starting in the root epidermis and progressing inwards with time. However, she regarded this as indicative of the development of aerenchyma in oxygen-deficient conditions - a suggestion that now seems to have been erroneous.

Holden (1975, 1976) first drew the attention of plant pathologists to the importance of root cortical senescence. Using both nuclear (Feulgen reagent) and cytoplasmic stains, he found that substantial parts of the cortex were non-viable in 3-4 weak old

wheat and barley roots grown in unsterile sand in the absence of pathogens at 15°C. The phenomenon was studied further by Henry & Deacon (1981) who termed it root cortex death (RCD), a term that will be used throughout this thesis.

RCD occurs in the absence of pathogens and even in the absence of microorganisms (Henry & Deacon, 1981). It has been observed in roots of small-grained cereals grown *in vitro*, in glasshouse and in field conditions (Holden, 1975; Deacon & Henry, 1981; Henry & Deacon, 1981), and similarly in a range of non-cereal grasses (Kirk, 1982; Kirk & Deacon, 1986). It has also been reported to occur in maize roots (Deacon *et al.*, 1986) but its pattern was different from that associated with the development of aerenchyma and the factors that govern it were also found to be different from those involved in aerenchyma formation.

RCD in cereals and grasses begin just behind the zone of living root hairs, where the epidermal cells begin to lose stainable nuclei, and it increases with distance from the root tip. The process starts in the epidermis and continues inwards until five of the six cortical cell layers of the seminal root axes are anucleate. The innermost cortical cell layer next to endodermis retains its nuclei, as does the stele, and there is also a detectable delay in senescence of the cortical cells around the bases of laterals. There is also a very small zone, less than 1 cm long, immediately below the grain where nuclei trend to persist in most cell layers of the cortex. The root apparently functions normally despite this tissue senescence because it extends at the tip and it can produce laterals with fully nucleate cortices. As they age, the laterals also show

cortical senescence, which follows the same pattern as in the root axes.

RCD occurs to a considerable extent. For wheat and barley grown in pathogen-free soil at 15°C, Holden (1975, 1976) reported that 65% and 41% respectively of the cortical cells were anucleate in the oldest region of the roots of 3 week old plants. Yet these roots appeared white and healthy and this suggests that the rates of cortical senescence in cereal roots had previously been underestimated. This general pattern and extent of RCD has been confirmed in many subsequent papers. As noted earlier, RCD probably accounts for much of the organic carbon known to be released from functioning cereal roots (Martin, 1977; Newman, 1985).

RCD is a genetically programmed phenomenon as evidenced by its occurrence in aseptic conditions, the fact that it always follows the same pattern, the finding that different cereal species have inherently different rates of RCD (but a similar pattern of RCD), and the finding that both varieties and chromosome-substitution lines have different rates of RCD. The rate of RCD is fastest in wheat, slower in barley, and slower still in oats and rye (Holden, 1975; Henry & Deacon, 1981; Deacon & Mitchell, 1985; Yeates & Parker, 1986). Such differences are also seen in field conditions (Henry & Deacon, 1981). The rate differs to a lesser degree between cultivars (Henry & Deacon, 1981) and is influenced by chromosome substitutions in wheat (Deacon & Lewis, 1982).

Nevertheless, growth conditions can affect the rate of RCD. Brown & Hornby (1987) reported differing effects of ammonium- and nitrate-nitrogen on the rate of RCD in wheat in gnotobiotic conditions. Gillespie & Deacon (1988) found that nitrogen deficiency and especially the lack of nitrate-N enhanced RCD in wheat seedlings

and wheat root pieces, showing that the mineral nutrients may exert direct, localised effects on root cell viability, in addition to having indirect effects through alterations of whole plant physiology. MacLeod *et al.* (1986) reported that RCD in wheat was influenced by phosphorus supply to soil, but interpretation of the results was complicated by possible effects of phosphorus on patterns of plant growth.

Other factors that have been shown to affect the rate of RCD include impedance of the root tip, which enhances the rate of cell death (Kirk & Deacon, 1986) and temperature (Smiley & Giblin, 1986). In the latter case, supra-optimal temperatures for growth of the grass *Poa pratensis* were found to accelerate RCD and also, unusually, to alter the pattern of cell death because this tended to occur first in cells near the stele. Microorganisms, however, were reported not to affect the rate of RCD of wheat grown in tubes of vermiculite (Henry & Deacon, 1981). Similarly, inoculation of cereal roots with the weak parasites *Phialophora graminicola* and *Microdochium bolleyi* was found not to enhance the rate of RCD (Henry & Deacon, 1981). These experimental studies contrast with the supposition of Martin (1977) that RCD, as evidenced by the release of organic carbon from roots, is accelerated by microorganisms. Aeration also was found not to have a major effect on the rate of RCD in maize adventitious roots (Deacon *et al.*, 1986). But these workers recorded the interesting phenomenon that RCD could occur simultaneously with aerenchyma formation in non-aerated roots in solution culture, RCD began in the root epidermis and progressed inwards with distance behind the root tips, whereas aerenchyma formation was associated with deletion of nuclei from the middle cortex and this (together with cell autolysis) progressed inwards

and outwards through the cortex with distance behind the root tips. In other words, RCD and aerenchyma formation were found to be under different control (the latter being a response to anoxic conditions) and also showed different patterns of nuclear loss in the root cortex.

It has been suggested that RCD occurs due to depletion of assimilates in cortical cells (Henry & Deacon, 1981). Root pieces placed on potato-dextrose agar, a sugar-rich medium, showed delayed RCD compared with those placed on water agar (Deacon & Lewis, 1986). Gillespie (1986) also found that exogenous glucose supplied to wheat root segments slowed the rate of RCD but did not prevent it.

The root cortices and coleoptiles of wheat and barley senesced more slowly in shaded than in unshaded conditions of seedling growth, whereas infection of leaves of barley by *Erisiphe graminis* had little effect on cortical senescence (Lewis & Deacon, 1982). In both treatments - shading and powdery mildew infection - the plants showed reduced growth of the root system and the authors suggested that poor root development reduced competition for assimilates within the root system despite the reduced total assimilate supply to the roots, resulting in a slower rate of RCD in the existing roots which were assessed for RCD. It seems that the pattern of RCD (starting from epidermis and proceeding inwards, and from younger to older root parts) is not affected by exogenous factors. Only exogenous kinetin, a growth regulator known to affect senescence in plant tissues, has been reported to delay RCD and change the normal polarity of cortical senescence from older to younger parts of root pieces (Gillespie, 1986). From all the above it seems that the phenomenon of RCD, once "triggered", can be somewhat delayed but not reversed with cytokinins or nutrient supply.

1.2.2. Root cortical senescence in dicotyledonous plants

In the roots of woody angiosperms and gymnosperms, and of most herbaceous dicotyledons, a periderm develops and the epidermal and cortical tissues exterior to it are eventually shed. The outer layer of the roots then comprises corky cells which mature and die. In addition to this Rogers (1968) and Rogers & Head (1969) observed that the new roots of fruit trees about 1-5 cm from the tip are white and succulent and bear root hairs. However, after a short period (1-2 weeks in apple in the summer months), the epidermis and the primary cortex begin to degenerate, turn brown in the area 7–10 cm from the tip and eventually are shed completely in the area 13-17 cm from the tip. The authors state that the amount of dry organic matter thereby added to the soil equals the amount of the organic matter in the remaining stele. Thus, about half of the dry matter produced by the new root up to that stage is returned to the soil and much of this provides substrates for soil microorganisms. Separation of dead or dying roots from the living portions of the root system eventually takes place, and protective layers of periderm develop on the base of the moribund roots (Reynolds, 1975). Very little is known about the physiology and early stages of these processes.

Addicot (1982), in a review of abscission, states that roots abscise bark, especially roots of woody perennial species. Many of these produce successive layers of bark, which rarely if ever builds up to a thick covering. The amount of tissue produced is usually small, and the outer layers are subject to decay. The death of the cortex in roots that undergo secondary thickening is not strictly equivalent to RCD in Gramineae which occurs in young root regions and progresses inwards towards the endodermis in the absence of

secondary thickening. Roots that do not develop much in the way of secondary thickening or periderm may still abscise the epidermis and the outer layers of the cortex as a result of cortical lysis. On such roots, a rhizosheath can form as sandy particles from the soil become cemented to the root hairs of the epidermis.

After the discovery of early root cortical senescence in graminaceous plants, dicotyledonous plants were examined with nuclear staining methods. However, there are only few such studies and the results are contradictory. MacLeod *et al.*(1986) reported the occurrence of RCD in rape (*Brassica rapa*) roots but not in subterranean clover after three weeks growth. Tommerup (1984), however, found no evidence of RCD in 6-week-old rape plants.

In older reports, the rootlets of perennial dicotyledonous plants, such as alfalfa (Jones, 1943), apple (Rogers, 1968), and strawberry (Nelson & Wilhelm, 1957; Wilhelm & Nelson, 1970), were found to function for a relatively short time and then senesce and die. Under favourable conditions of plant growth they are replaced. Reynolds (1975) first expressed the view that most of the lateral rootlets of very small diameter may be determinate in growth and duration. This senescence and death of whole root members is, however, quite different from root cortex senescence in the Gramineae.

There are also more recent reports of whole root senescence and death, from direct observations and from estimates of total root mass. Huisman (1982) states that the incidence of root death for field-grown cotton was quite low during the early part of the growing season. Its magnitude approached or equalled that of root growth during the last two thirds of the growth season. But towards the end of the season, less than one fifth of the cumulative root

length was still alive. It seems that root turnover occurs mainly in roots of the smaller diameter. This makes quantitative studies of root turnover in soil extremely difficult, because these roots are decomposed very fast. The overall large increase in root mass observed for many plants in fumigated soils, compared to non-fumigated soils, suggests the involvement of the soil microflora in a major part of root death. However, it is not clear whether the soil microflora directly causes death of the roots or only accelerates the death of senescing rootlets.

In forests and orchards a considerable proportion of small roots die each year. Implications of this for nutrient-cycling processes have been considered by Fogel (1983). It is calculated that in every hectare of forest 1100 kg of root material becomes non functional and dies each year. The quantity of roots dying each year can approach one-tenth of the quantity of leaves, branches and bark falling from the trees of a forest (Head, 1973). Death of the absorbing rootlets of trees has been related to a number of factors, including seasonal variations in moisture and the actions of soil microorganisms.

Reynolds (1975), however, considers that it is an adaptive phenomenon, whereby trees shed the ultimate rootlets at the end of the main growing season and thus avoid the need to expend energy in maintaining them. In this respect, "root shedding" in dicotyledonous plants may be functionally equivalent to RCD in the Gramineae: a plant may maintain living root cells only for as long as it needs to do so. Atkinson (1983) has argued that the uptake of water (at least) can occur over most or all of a root system - even in suberised regions of the roots of fruit trees. Uptake of water and the more mobile mineral nutrients can similarly occur through the

older regions of cereal and grass roots (Clarkson *et al.*, 1971). So in both "systems" young living root regions may be needed only for uptake of the less mobile nutrients such as calcium and phosphorus from soil zones that have not been depleted of these minerals; thereafter, the cost of maintaining the viability of the young root regions may outweigh the advantage of retaining these.

1.3. Parasitic fungi associated with cereal and grass roots

The significance of root tissue senescence for colonisation by soil-borne microorganisms has been studied mainly in the Gramineae and especially in cereals. RCD seems to provide a major source of nutrients for rhizosphere bacteria (Van Vuurde & Schippers, 1980; Deacon & Lewis, 1982; Deacon, 1987) the population of which increases markedly in regions of cortical senescence.

RCD similarly seems to account for the activities of a range of fungi that are commonly found as parasites on roots of the Gramineae (Deacon, 1987). Such fungi include *Phialophora graminicola* (Deacon) Walker, *Phialophora zeicola* Deacon & Scott, *Gaeumannomyces graminis* (Sacc.) von Arx & Olivier var. *graminis*, the pathogenic *G. graminis* var. *tritici* Walker (*Ggt*), causal agent of the take-all disease in cereals and *Microdochium bolleyi* (Sprague) de Hoog & Hermanides-Nijhof.

Von Arx (1981) recently reviewed the taxonomy of *Microdochium* and *Idriella*, and placed *M. bolleyi* in the later genus. However, the taxonomy of this variable fungus is confused and, pending full acceptance of von Arx's treatment by pathologists, the fungus is here termed *M. bolleyi*.

The take-all fungus (*G. graminis* var. *tritici*) grows externally along the roots as dark runner hyphae from which narrower hyaline hyphae penetrate the root

cortex. After the cortex is invaded, the fungus penetrates the vascular system where it causes dark brown discolouration. In the late stages of infection the vascular lesions occur abundantly in the basal zones of the root system, the phloem is disorganised, the uninfected apical portion of the root no longer receives a supply of photosynthates, ceases growth and dies.

P. graminicola has a similar growth habit to that of *G. graminis* growing along root surfaces and occasionally penetrating the cortex, but it never penetrates the vascular system to cause disease. It is a weak parasite rather than a pathogen (Holden, 1976).

In fact, Holden (1975, 1976) first recognised the importance of RCD during studies on *P. graminicola*. He showed that the fungus penetrates cereal roots only to the depth at which the cortex has died naturally. Subsequent work by Deacon (1980) confirmed this for naturally infected wheat roots from a field site where wheat had been grown after a grass ley (on which the population of *P. graminicola* had become established). More recently, Deacon & Lewis (1986) showed the same thing for invasion of pieces of sterile excised root; in a comparison of *Ggt* and *P. graminicola* in these conditions the degree of invasion of roots by *P. graminicola* was determined by the rate of cortical senescence whereas *Ggt* could invade even fully living cortical tissues. *P. zeicola* (Deacon & Scott, 1983) and *G. graminis* var. *graminis* (Deacon, 1987) seem to be intermediate in their degree of invasiveness and thus are weakly pathogenic.

M. bolleyi is commonly isolated from the rhizosphere and haulm base of cereals and grasses. Sprague (1948) isolated it from 121 graminaceous species in the USA, and several reports confirm its ubiquitous occurrence in the rhizosphere of these plants (Salt,

1977; Reinecke *et al.*, 1979; Waller, 1979; Deacon, 1980; Murray & Gadd, 1981; Reinecke & Fokkema, 1981). It has also been reported in the rhizosphere of non-graminaceous plants (Domsch *et al.*, 1980; Black & Brown, 1986). It is classed as a "minor pathogen" which implies that it causes yield losses either alone or in association with other pathogens (Black & Brown, 1986). *M. bolleyi* seldom causes serious damage in artificial inoculations unless it is present on seedling roots at an overwhelming inoculum level (Fitt & Hornby, 1978; Kane & Smiley, 1987; Kirk & Deacon, 1987b). In artificial inoculations Waller (1979) found that *M. bolleyi* had no significant effect on growth of wheat or barley, while Fitt & Hornby (1978) observed damage in roots of wheat but the plants soon recovered and there were no significant effects on subsequent growth. Environmental conditions and host vigour largely determine its ability to cause disease (Colhoun, 1973; Fitt & Hornby, 1978; Reinecke *et al.*, 1979; Salt, 1979).

In glasshouse experiments the roots of wheat and cocksfoot grass (*Dactylis glomerata*) were inoculated with *M. bolleyi* and the pattern and rate of invasion were strongly correlated with the rate of RCD (Kirk & Deacon, 1987b). The fungus did not, however, enhance this cortical senescence, confirming an earlier finding by Henry & Deacon (1981). *M. bolleyi* forms aggregates of dark brown chlamydospore-like cells within the root cortex of host plants, and it can be recognised by these aggregates (Salt, 1976, 1979). Because the fungus is frequently isolated from plants, there has been recent interest in its possible interactions with pathogenic fungi in the same plants. Although the fungus has usually been categorised as a "minor pathogen", some reports suggest that *M. bolleyi* may be beneficial by controlling or reducing the activities of other fungi.

Salt (1979) cited unpublished work by I.M. Waller, that *M. bolleyi* can decrease damage done by *Pythium arrhenomanes* Drechs. on cereals. It is also reported that *M. bolleyi* antagonises *Fusarium* spp. in the haulm base of wheat and rye, and can reduce infection by *Pseudocercosporella herpotrichoides*. (Fron.) Deighton, the eyespot fungus (Reinecke et al., 1979; Reinecke & Fokkema, 1981). Most recently, *M. bolleyi* was shown to act as a biocontrol agent of take-all and to reduce the degree of root colonisation by the non-pathogenic *P. graminicola* (Kirk & Deacon, 1987a)

1.3.1. Implications of root cortical senescence in biological control of take-all

P. graminicola is a biological control agent of take-all (reviewed by Deacon, 1981; Wong, 1981). It is believed that the natural population of *P. graminicola* in British grasslands delays the establishment of severe take-all in subsequent cereal crops (Deacon, 1973; Slope *et al.*, 1978) and also can control the take-all patch disease of sport turf, caused by *G. graminis* var. *avenae* (Turner) Dennis (Deacon, 1973).

In field trials in Australia, two weak parasites, *G. graminis* var. *graminis* and a *Phialophora* sp., both of which grow as dark runner hyphae on roots and are closely related to *P. graminicola*, were found to give significant control of take-all of wheat when their inocula were added to soil (Wong & Southwell, 1979). Take-all patch of turf grass was similarly controlled by these fungi in glasshouse trials (Wong & Siviour, 1979). Hypovirulent isolates of the take-all fungus itself have also been shown to control take-all of wheat in field trials in France (Lemaire *et al.*, 1977). *M. bolleyi* can also control infection by *Ggt* (Kirk & Deacon, 1987a) repeated above

and in experimental studies can "control" the extent of growth of *P. graminicola* (itself a biocontrol agent of take-all) on cereal roots (Kirk & Deacon, 1987a).

Various explanations have been advanced for all these findings. For example, Lemaire *et al.* (1977) implicated viruses of hypovirulent strains of *Ggt* in the biocontrol mechanism by these strains, but this has not generally been supported by later work (Rawlinson & Buck, 1981). Speakman & Lewis (1978) implicated induced host resistance by *P. graminicola* in biocontrol of take-all by this fungus. However, this also is not compatible with other work. For example, *P. graminicola* did not induce the supposed host resistance mechanisms (stelar lignification reactions) in maize or rye-grass roots (Speakman *et al.*, 1978) and yet *P. graminicola* can control *Ggt* in roots of these plants (Deacon, 1981). Also, at appropriate relative inoculum levels, the take-all fungus can "control" the degree of cortical infection by *P. graminicola* which does not enter the stele and does not, in any case, usually invade living host cells (Deacon, 1981). The most likely mechanism of control was suggested by Deacon & Henry (1981) and has been supported by subsequent studies (Kirk, 1984; Kirk & Deacon, 1987a). It is that all these fungi can compete with one another for colonisation of senescing root cortical cells because all of them are specialised to exploit RCD. In arriving at this explanation, Deacon & Henry (1981) had to account for the fact that *Ggt* can normally invade fully living cortices and thus, theoretically, should not be dependent on RCD and should not be controlled by weak pathogens or weak parasites. But such studies are based on the use of large inocula of *Ggt* which provide a food base to support infection. At critically low inoculum levels (e.g. from ascospores) *Ggt* cannot infect roots

directly to cause disease (Brooks, 1965; Kirk, 1984). Then it needs to undergo a "feeding stage" on the roots (Brown & Hornby, 1971) before it invades the stele, and in this feeding stage *Ggt* is thought to depend on RCD to establish a food base for subsequent infection.

Consequently, any fungus that is adapted to exploit RCD could, if previously established on the roots, interfere with the ability of *Ggt* to amass a sufficient food base for infection.

This explanation, as noted by Deacon (1981, 1987), is consistent with all the available evidence. It helps to explain, for example, how *Ggt* can interfere with the growth of *P. graminicola* on roots, and vice-versa. It explains why the outcome of an interaction between any two such fungi (e.g. *P. graminicola* and *M. bolleyi*) is determined by their relative inoculum levels and by which fungus is established first on a root system. In practical terms, it explains why all of the control agents of *Ggt* are effective only in the early stages of a cereal sequence, preventing or delaying the build-up of damaging populations of the pathogen but being unable to control the pathogen once this is well established in a site.

1.4. Parasitic fungi associated with senescing roots in dicotyledonous plants

Since very little is known about natural (non-pathogen-induced) senescence in roots of dicotyledonous plants, our knowledge of the parasitic microflora associated with naturally senescing roots is very poor and not well documented. There is scattered information that senescence may play some role in root disease like that caused in strawberry by *Cylindrocarpon destructans*, which alone or in association with *Fusarium oxysporum*, *Phialophora* spp., and certain

actinomycetes and bacteria gains access to the main root tissues through the vascular connections with dead rootlets (see Salt, 1979).

Of interest in relation to the work in this thesis is the association between tomato and its pathogen *Pyrenochaeta lycopersici*, which causes the disease known as brown root rot or corky root. Although neither host nor pathogen has previously been studied in relation to natural senescence there are aspects of this disease that indicate a possible involvement of tissue senescence. The characteristic symptoms of corky root usually appear late in the growing season (Ebben, 1973), on older roots, while other symptoms such as root browning appear earlier on young feeding roots and as darker lesions in which the cortex cracks on older, larger roots. The infected young roots soon rot and the fibrous root system of plants in infested soil is considerably smaller than that of plants in non-infested soil (Last *et al.*, 1966). Yield losses are more frequently related to the brown root rot phase than to root corkines. Early infections are more devastating than those occurring later.

P. lycopersici is a grey slow-growing fungus that rarely produces pycnidia. Although it was often isolated from diseased tomato roots only in 1964 was it recognised as a form of *Pyrenochaeta* (Gerlach & Schneider, 1964). Brown root rot had previously been thought to be caused partly by other fungi, notably *Colletotrichum coccodes* (Wallr.) Hughes but this fungus is a secondary invader of tomato roots (Last & Ebben, 1966). *P. lycopersici* forms microsclerotia in culture and in the tissues of roots in the region where the interior of the corky zone of the lesion merges with the apparently normal cortical tissue (Preece,

1964; Ball, 1979). It causes serious losses in tomato yields when present at a high inoculum level in soil. This happens when tomato is grown in successive years in soil, resulting in a progressive increase in soil inoculum (Last *et al.*, 1969). The fungus also causes disease symptoms on roots of other species of the Solanaceae, both in nature and following artificial inoculation (Termohlen, 1962; Taylor *et al.*, 1971; Malathrakis *et al.*, 1983; McGrath & Campbell, 1983). Its host range extends to non-solanaceous plants such as melon (Risser & Lougie, 1968) and cucumber (Termohlen, 1962), and it has been isolated from roots of many other plant species on which it does not cause disease. For example, Grove & Campbell (1987) were able to isolate it on a semiselective medium, from spinach and sufflower, and from squash and *Datura stramonium* following artificial inoculation.

Fungi such as *P. lycopersici* seem to be ubiquitous in plant rhizospheres but their ecological relationships remain largely unknown. Salt (1979) mentions that several grey sterile fungi differing in cultural characteristics and growth rate were isolated from winter wheat root segments that had been surface-sterilised and plated on PDA. The root axes from which these fungi were isolated showed no symptoms, and the fungi lacked pathogenicity to wheat. Grey sterile fungi, also called *Mycelium radicis atrovirens*, have been associated with root diseases in strawberry (Wilhelm *et al.*, 1969). It is not known if all these grey sterile fungi reported from wide range of hosts are the same or closely related species. The likelihood is that they are different, one from another, as Hall (1987) found in a detailed analysis of the sterile fungi from cereal roots.

1.5. Aims and scope of work in this thesis

The general aim of the work in this thesis was to further understanding of the relationship between non-pathogen-induced senescence of root cortical tissues and the ecology of parasitic root-colonising fungi. The study comprised four main areas, outlined below. They were selected because they were complementary and might, together, indicate the degree to which root cortical senescence influences the behaviour and distribution of root-parasitic fungi.

1. Study of factors influencing RCD, with particular regard to factors known to influence senescence in other plant systems.
2. Study of the invasion of senescing root cortical tissues by *M. bolleyi* and *P. lycopersici*, in relation to manipulation of cortical senescence by experimental treatments.
3. Study of the role of spores of *M. bolleyi* in colonisation of the roots of wheat.
4. Investigation of the possible existence in tomato roots of a phenomenon similar to RCD in cereals, tomato being taken as a representative dicotyledonous plant.

2. Materials and Methods.

Because of the diverse nature of the experiments in this thesis, most experimental details are given in the appropriate sections.

2.1. Fungal cultures

Fungal cultures used in this study were as follows. *Microdochium bolleyi* strains T560, J10, T560R1 and J10R1. Strain T560 was isolated by Dr. G.A. Salt from barley at Rothamsted in 1976; strain J10 was isolated by Dr. Jane Kirk from cereals near Edinburgh, strains T560R1 and J10R1 were mutants tolerant of benzimidazoles, produced from T560 and J10 respectively, as described in Section 6.2. *Pyrenochaeta lycopersici* was isolated in 1988 from tomato plants in a commercial glasshouse in Skydra, Greece. *Gaeumannomyces graminis* var. *tritici*, isolate NP2, was isolated from wheat in 1986 and strain BG1 was isolated from barley in Lincolnshire in 1988.

All were regularly sub-cultured on agar plates at 25°C, the medium being Oxoid potato dextrose agar (PDA) or, in the case of *Pyrenochaeta lycopersici*, home-made V8 agar. In addition, the fungi were stored at 4°C as 5 mm agar discs cut from cultures and submerged in distilled water in screw-cap McCartney bottles. These cultures were renewed every six months. *P. lycopersici* was inoculated into moist sterile soil, left to dry and stored at -25°C.

P. lycopersici produced pycnidia in 15-25 days when grown on home-made V8 agar at 20°C under continuous illumination with "black light" (Philips TL 20W/08 F20T 12BLB). Pycnidiospores from newly produced pycnidia (not more than 15 days old) were used in the experiments.

2.2. Agar media

Potato dextrose agar (PDA, Difco) was prepared according to the manufacturer's instructions. V8-agar was used for culture and

sporulation of *P. lycopersici* (McGrath & Campbell, 1983). Home made V8 juice is better than the commercially available product for sporulation of this fungus (Hockey, 1984). It was prepared as follows. Beetroot, carrot, celery, lettuce, parsley, spinach, tomato and watercress (10 g each) were blended with 1l water, simmered for 30 minutes and strained through double-layered muslin; 2% agar was added and the medium was autoclaved.

Selective medium for isolation of *P. lycopersici* was prepared by adding penicillin, streptomycin, chlortetracycline and Bayleton, each at 100 μg ml^{-1}, to PDA that had been autoclaved and cooled to 50 °C.

Selective medium for isolation of *M. bolleyi* strain T560R1 (benzimidazole-tolerant) comprised 50 μg ml^{-1} streptomycin, 50 μg ml^{-1} chlortetracycline and 5 μg ml^{-1} Carbendazim in quarter-strength PDA or in water agar.

White's minerals agar was used as an incubation medium for root pieces. Its constituents were (μg/ml): $MgSO_4 \cdot 7H_2O$, 731; $Na_2SO_4 \cdot 10H_2O$, 453; $Ca(NO_3)_2 \cdot 4H_2O$, 288; KNO_3, 80; KCl, 65; $NaH_2PO_4 \cdot 2H_2O$, 21.5; $MnCl_2 \cdot 2H_2O$, 6; $ZnSO_4 \cdot 7H_2O$, 2.65; H_3BO_3, 1.50; KI, 0.75; $CuSO_4 \cdot 5H_2O$, 0.13; H_2MoO_4 0.0017; ferric ethylenediaminotetra-acetate (EDTA), 8. This medium was solidified with 2% agar.

2.3 Cultivation of plants

The plants used in this study were as follows. Wheat (*Triticum aestivum* L., cv. Avalon), barley (*Hordeum vulgare* L., cv. Igri), oat (*Avena sativa* L., cv. Commander), rye (*Secale cereale* L., cv. Danko) and tomato (*Lycopersicon esculentum* Mill. cv. ACE 55 VF). Seeds were surface-sterilised by immersion in 95% ethanol for 5 sec., followed by a washing in sterile distilled water, and then soaked in a saturated solution of calcium-hypochlorite for 20 minutes (cereals)

or 10 minutes (tomato). The seeds were washed twice more in sterile distilled water, and allowed to germinate overnight on sterile moist filter paper at room temperature.

Plants were grown in a glasshouse at a mean temperature of 20°C. Natural light was supplemented with fluorescent tubes (Philips "warm-white" and "daylight") supplying 2000 lux at plant height, and mercury vapour lamps supplying 4000 lux, for 15h each day.

In one experiment (section 4.8), tomato cuttings were used. These were obtained from 5-6 week old tomato plants raised from seed. The stems were cut transversely with a razor blade 0.5 cm above and 5 cm below a leaf petiole. The cuttings thus obtained were planted in cups with perlite, under transparent covers (propagators) and they rooted within one week.

2.3.1. Plant growth containers

Plastic drinking cups (c. 7 cm diam., 8 cm deep) with perforations in the bases, were filled with horticultural perlite for use in most experiments. Where a greater rooting depth was required, this was provided by 30 cm long rigid perspex tubes, 5 cm diam., or 40-75 cm lengths of "layflat" flexible polyethylene tubing, 5 cm diam. The perspex tubes were sealed at their bases with nylon netting and the bases of the polyethylene tubes were closed by making a knot, and V-shaped cuts were made with a scalpel to provide drainage. They were filled with perlite and covered with black polyethylene sheeting to exclude light from the roots. The polyethylene tubes were suspended vertically from a metal framework or were laid against sloping, transparent corrugated perspex as appropriate for particular experiments.

2.4. Preparation of sterile seedlings and root pieces

Seeds of wheat, barley, rye, oats or tomato were surface-sterilised by immersion in 95% ethanol for 4-5 seconds and then soaked in 0.05% $HgCl_2$ solution, the seeds of cereals for 2 min. and of tomato for 1 min. Seeds were washed by shaking them in seven or eight changes of sterile distilled water (SDW), for 10 minutes each time, dried with sterile blotting paper and plated on potato dextrose agar (PDA), 15-20 seeds per dish. After incubation for 48 h at 25°C in darkness, those showing contamination (usually <1%) were discarded and the rest were transferred to Petri dishes containing Oxoid No. 3 agar (2%) and mineral salts as in White's (1943) medium. There were 4-8 seeds per dish, and they were incubated for a further 2 days (cereals) or 4 days (tomato). In all cases the seeds were aligned and placed so that the emerging root grew towards the centre of the dish, and the dishes were sloped almost vertically. So, all roots were growing in the same direction across the dishes, and this facilitated the collection of uniform root pieces for use in the experiments. The apical 2 cm of a root, including the tip, was cut with a scalpel and transferred to a new mineral agar plate as above, maximum five root pieces per plate. At sampling, replicate root pieces were taken always from different Petri dishes. Because the young roots and especially the tomato roots are very fragile, in the later experiments a variation of this procedure was used: the cut root piece was not removed but left on the original agar plate and the rest of the seedling was removed. In cereals the root pieces were taken from the first-formed seminal root axes, one root from each seedling. Each 9 cm Petri dish contained 15-20 ml agar medium. The dishes were incubated at 25°C in darkness. Variations of these methods were used according to the needs of individual experiments, described where appropriate.

2.5. Assessment of cell senescence and death

Sectioning

A freezing microtome was used for sectioning of roots. In order to reduce cell damage caused by the production of ice crystals, root segments were pretreated in 2% (v/v) glycerol (Knox, 1970). A drop of dextrin-syrup (Steedman, 1960) was placed on the precooled block holder and just as it was about to freeze, the root segment was rapidly transferred to the block holder and embedded in the freezing drop, so it was mounted and frozen in one operation. Then a precooled knife (-25°C), was placed on the microtome and the sections were cut rapidly. The sections were picked from the knife and stained or treated as follows.

Acridine orange.

For assessment, the laterals were removed, then the roots were hydrolysed in 3% HCl in 95% ethanol (15 min at room temperature) washed twice (2 min) in phosphate-citrate buffer of pH 3.8, stained in 0.001% acridine orange in the same buffer (10 min at room temperature) and rinsed twice in buffer. Whole roots or root pieces were then mounted in buffer on microscope slides and examined under a Leitz "Orthoplan" transmission fluorescence microscope, usually at 100 x magnification, with exciting filters BG 12 and BG 38 and suppression filter K 510. The nuclei fluoresced greenish yellow and could be seen through the depth of the cortex. Roots were scanned by microscope fields (x 100 magnification) each 1.8 mm diam. and the presence or absence of nuclei was recorded for each of the usually six cortical cell layers outside of the endodermis of cereal roots, for successive microscope fields along the root axes. Anucleate cells were considered dead.

Neutral red - plasmolysis

Fresh root pieces were placed in a 0.02% solution of neutral red dissolved in 0.6 M KNO_3 solution, containing 10 mM $CaCl_2$ and buffered to pH 7.5 with 0.02 M phosphate buffer; after 30-60 min in the stain the roots were placed in fresh KNO_3 solution as above but without the stain for 1-2 min, mounted on microscope slides in this solution and examined at x 100 magnification. Stained and plasmolysed cells were considered alive.

Fluorescein diacetate (FDA)

For the assessment of viability of root tissues with fluorescein diacetate the method of Widholm (1972) was used, but modified by using White's mineral nutrient medium (Section 2.2) for the final dilution of FDA. A stock solution of 5 mg FDA ml^{-1} acetone was stored in a freezer and diluted in White's medium to give a final concentration of 0.01% FDA. This diluted solution was usable for only a few hours. The root pieces were placed in it for 5-20 min then placed on microscope slides, mounted in distilled water and observed immediately under an incident fluorescence microscope with exciting filter BG 12 and barrier filter 47.

The viability of cells was assessed by their ability to accumulate fluorescein, due to enzymic cleavage of the ester linkage of the acetate. This dye yields greenish yellow fluorescence on excitation with UV light and is detectable in low concentrations. The free fluorescein can diffuse from cells, so the concentration of fluorescein in a cell depends upon the cells' capacity to replace the lost dye. Dead cells cannot cleave the ester and so they do not fluoresce.

Cytochemistry for acid phosphatases

Freeze microtome and free-hand sections of fresh unfixed roots were cut with a new razor blade and used immediately for cytochemistry.

Acid phosphatase activity was determined by the method of Gomori (1952) as modified by Miyayama *et al*. (1975). Sections were incubated in 1.5 mM para-nitrophenyl phosphate (p-NPP-Na_2) together with 2.0 mM lead nitrate in 0.05 M acetate buffer at pH 5.8. Sections were incubated at 37°C at 5 min intervals from 1 -60 min in order to determine the initial response times for each tissue.

β-glycerophosphatase activity was determined using the same method as improved by Pearce (1960). In this method the sections were incubated in 10 mM sodium β-glycerophosphate in 0.05 M acetate buffer (pH 5.0) and 4 mM lead-nitrate, at 37°C. As controls, sections were incubated in the absence of the substrate or in the presence of 10 mM NaF. After incubation the sections were washed briefly with distilled water and immersed in dilute yellow ammonium sulphide for 1-2 minutes, washed again in distilled water, mounted in water and observed under a light microscope.

DNAase activity

A 5% solution of gelatin (Oxoid) prepared by heating in an oven at 55°C was mixed with an equal volume of 3% DNA (Sigma, type III, sodium salt) at the same temperature. The mixture was passed several times through a fine needle of a syringe in order to be of homogeneous viscosity. Three drops of the gelatin-DNA mixture were placed on a glass slide, rapidly spread with the tip of a pipette over a surface of about 2.5 x 4 cm and the excess was allowed to

drain by placing the slide vertically on a piece of filter paper. The film was allowed to dry at room temperature in a vertical position. Then gelatin-DNA films were fixed by immersing the slides for 16h at 5°C in 20% formaldehyde solution neutralised with an excess of calcium carbonate. The fixed films were washed in three baths of distilled water, 5 min each time, and allowed to dry at room temperature.

The gelatin-DNA films were kept in a water-saturated atmosphere in a Petri dish at 20°C. Freshly cut frozen root sections were transferred to the films using a fine brush so that they thawed and adhered to the film. They were incubated for 1, 2, 4, 8 and 16 minutes, then flushed off the films with water and the slide was immersed again in neutral 20% formaldehyde solution for 1 hour at room temperature to stop the enzyme action. The film was washed in three baths of distilled water, 5 min each time, and allowed to dry. Then it was stained with 0.2% toluidine blue for 5 min, followed by three 5 minute washes in distilled water and allowed to dry again. The slides were observed under a microscope. The film areas that failed to stain represented sites of enzymic hydrolase activity.

2.6. Assessment of lignification and of autofluorescence of cortical cells

The extent and intensity of lignification of cortical cell walls and the occurrence of lignitubers (papillae) that developed in response to fungal invasion were assessed by staining root sections with phloroglucinol-HCl (Bradbury, 1973). The sections were incubated for 5-10 min in a solution of 5% phloroglucinol in 75% ethanol, then this was allowed partly to evaporate and the root tissue was flooded with 5 M HCl. Roots were mounted in 5 M HCl and

examined under a compound microscope for the presence of red-stained lignified cell walls or papillae. Autofluorescence was assessed by observing the sections in a fluorescence microscope with exciting filter BG1 and barrier filter 460.

Ten sections from each sample were examined and each cortical cell layer was scored for lignification or yellow autofluorescence of cells in each layer, using categories "none", "some", "most" cells showing these features. Finally the median was taken for each layer in each sample.

2.7. Assessment of fungal invasion of roots

The method of Koske & Gemma (1989) was used for staining of mycelium in sections of roots. The sections were heated in 2.5% KOH solution for 10 min at 90 °C, washed in water and left in 1% HCl overnight, then stained in acidic glycerol containing 0.05% trypan blue (Sigma) for 10 min at 90°C, destained and mounted in acidic glycerol. Acidic glycerol was prepared with 500 ml glycerol, 50 ml 1% HCl and 450 ml water.

2.8. Measurement of total root length

The length of root systems was measured using Newman's (1966) method as modified by Giovannetti & Mosse (1980). Roots were spread in a uniform single layer in a tray with a grid square of lines 1.27 cm apart and viewed with a stereoscopic microscope. The number of intersections between the grid lines and the centre lines of the roots represented the total length (cm) of roots in the tray. To ensure adequate precision, at least 200 intersections were routinely counted. For very large samples, subsamples only were measured in this way. To give adequate counts in small samples, trays with denser grids were used.

3. Assessment of root cortical death in sterile roots attached to, or detached from, seedlings and its implications for parasitic fungi

3.1. Introduction

In this part of the thesis, roots attached to young sterile seedlings or excised root pieces were used in studies of senescence and susceptibility to invasion by fungi.

Excised plant organs like leaves, flowers and fruits have been extensively used by plant physiologists in studies of senescence, partly because excision from the mother plant enhances the rate of senescence and thus facilitates these studies and partly because the experiments can be done in better-defined conditions than on whole plants, so factors that may interfere in a direct way or indirect way can be distinguished.

Senescence of root tissues has not been studied as intensively as that of other parts of plants. There is some information from research on roots grown *in vitro*, but most of this involves the activity and viability of the meristems and their ability to maintain growth. Little information exists for the fate of the differentiated root tissues behind the growing apex, and especially of the cortex, as the root ages.

The study of natural senescence of root cortices in roots attached to plants is often complicated, because environmental factors may influence RCD in a direct and localised way but can also influence the growth of the plant as a whole and the partitioning of assimilates to different parts of the plant (Lewis & Deacon, 1982). In order to overcome these problems, excised roots of cereals were used for studies of the process of senescence. Although there are problems in the use of excised roots - for example, the root is not receiving nutrients from the plant - yet several workers have found

obvious resemblance in the way the root cortex senesces in excised cereal roots and in roots attached to the plant; for this reason, root pieces have been used in studies of senescence and invasion by fungi (Kirk, 1984; Deacon & Lewis, 1986; Gillespie, 1986; Gillespie & Deacon, 1988). RCD in excised root pieces of cereals can be enhanced or delayed depending on the constituents of the medium used to support the root pieces, but always it follows the same pattern as in roots attached to plants (Gillespie, 1986).

The work in this section investigated cortical cell death in sterile roots attached or excised from seedlings, maintained on solid media under a variety of conditions. It parallels, in part, work on whole plants described in Section 4. The form of the experiments was based largely on the previous studies cited above. Solid rather than liquid supporting media were used, because the work of Deacon & Lewis (1986) and of Gillespie (1986) had shown that agar media were satisfactory for incubation of root pieces and that variations in agar media resulted in different rates of RCD. The use of agar-based media was also advantageous for studies involving localised inoculation of roots with fungi. Agar with inorganic nutrients but without organic ingredients (except Fe-EDTA) was used as the supporting medium. The composition of the mineral nutrients was as in White's medium (Section 2.2) which is used for growth of roots *in vitro*. Root pieces without tips were used in some experiments because it was previously shown that these did not extend whereas root pieces with tips continued to grow on agar, especially in the presence of sugar, complicating the interpretation of results. However, roots attached to young seedlings and root segments with both intact and excised tips were used in most of the subsequent experiments.

In the first part of this section, methods for assessment of senescence in root cortices of wheat and tomato were evaluated, and a comparative study of senescence in excised roots of wheat and tomato was done. The senescence of the better-known system of the wheat root cortex was compared with that of the cortex of tomato roots, taken as representative of dicotyledonous plants.

Because there was a need for an easy and accurate assessment of senescence and death in roots of both species, several methods were screened in preliminary experiments. Three of them that are routinely used to assess viability of cells, involving the use of neutral red followed by plasmolysis of cells (Basham & Bateman, 1975), acridine orange (Henry & Deacon, 1981) and fluorescein diacetate (Widholm, 1972) were evaluated in the first experiment. The cytochemical assessment of the distributions of acid phosphatase and of DNAase activities in root cortical tissues were also examined in an attempt to find a cytochemical marker of the early stages of senescence. The reason for this was that the loss of the specific staining properties of the nucleus in the cortical cells of cereals suggests a loss of the nuclear DNA and possibly the involvement of DNAases. Further, acid hydrolases have been localized in a number of plant cells that undergo differentiation, such as those forming the xylem vessels. Since this differentiation results in death of the cells involved, these early stages of differentiation represent the early stages of a programmed senescence and death, perhaps like those occurring in the cereal root cortical cells.

By the use of the Gomori (1952) technique for β-glycerophosphatase, Gahan & Maple (1966) demonstrated by light microscopy the occurrence of lysosome-like particles in the

undifferentiated meristematic cells of the root tips of *Vicia faba* L. They showed that the onset of cell differentiation leading to the formation of protoxylem vessels, which involves the death of the cells involved, was accompanied by a change in the location of these hydrolases from particulate sites to the cytoplasm in general. Similar observations to those of Gahan's group have been made in studies of the death of root cap cells, another example of programmed cell death, when the lysosomal location of acid phosphatase gave way to a diffuse cytoplasmic location at the onset of autolysis (Berjak & Villiers, 1970). It was also shown that widespread disorganisation in the compartmentation of cells accompanied the process.

In the next six experiments the effects of various factors on RCD were studied in roots of wheat seedlings or in root pieces, in order to investigate similarities and differences from other senescing plant systems. Excision of the shoot, seed or root tip of seedlings and incubation of root pieces on media containing factors known to affect senescence in other plant systems were tested. These factors included different combinations of sucrose and mineral nutrients applied at the basal or apical region of the root pieces, plant growth regulators, silver and cobalt ions, free radical scavengers, ascorbic acid and cycloheximide. From these experiments it was thus hoped to determine the main nutritional and physiological factors that might influence RCD, in the absence of complications arising from the use of whole plants.

In the last three experiments, root pieces or roots attached to seedlings of cereals or tomato were inoculated with parasitic or pathogenic fungi for studies of invasion of their cortices and the responses of host cells to invasion, in parallel with senescence.

3.2. Senescence of excised roots of wheat and tomato: methods for assessment of cell viability

In the main part of this experiment excised root pieces of tomato and wheat were incubated on mineral nutrient agar (MA). After 0, 5, 10 and 15 days' incubation, five root pieces from each treatment were sampled, the root pieces being taken from five different dishes. They were assessed for viability of their cortical tissues, using acridine orange (AO), neutral red (NR) combined with plasmolysis, and fluorescein diacetate (FDA, Section 2.5).

In preliminary trials it was found that root pieces assessed previously for viability with NR-plasmolysis or FDA could be destained in 70% alcohol for some hours and then assessed with AO for the presence of nuclei. This enabled comparison of the results from the different methods for the same parts of each root. To facilitate this, the basal end of each root piece was attached to a glass slide with a small amount of instant glue (Loctite "Super Glue" based on cyanacrylate). After staining, each root piece was assessed under a microscope (X100 magnification) along its length and each microscope field (diameter = 1.8 mm) was scored for the number of living cell layers. The seminal root axes of wheat had usually six cortical cell layers (including the epidermis) outside of the endodermis, and the tomato roots usually five cortical cell layers but sometimes six layers.

A cell was considered alive with the NR-plasmolysis technique if it could plasmolyse and the contracted protoplast was stained red or pink and easily could be distinguished from the surrounding cell walls. With the FDA stain the whole cell fluoresced under UV microscopy when it was alive. With the AO stain, a cell with a stained fluorescing nucleus was considered alive.

3.2.1. Results

Table 3.1 shows the number of living cortical cell layers assessed by staining with AO, NR or FDA for different times of incubation on MA medium. Each value represents the mean with SE averaged across all microscope fields. Separate root pieces were used for each staining method. But the pieces assessed with FDA and NR-plasmolysis were further assessed with AO, so the means and standard errors for 10 root pieces subjected to this "double staining" are shown in parentheses in Table 3.1.

All cortical cells of both wheat and tomato were nucleate on day 0 (just after excision) when assessed with AO and also were assessed as being alive by the other two techniques (e.g.Fig. 3.2). However, some root hairs were assessed as dead, probably because of damage during handling. Other findings in the experiment are considered under separate headings as follows.

Comparison of AO and NR-plasmolysis in senescing wheat root pieces

By the fifth day of incubation a mean of more than two cell layers of wheat roots failed to plasmolyse. All the presumably dead cells were in the epidermis and outer cortex. The same cortical layers were anucleate when assessed with AO. Cell death had advanced further inwards by the tenth day of incubation, such that only cells in one cortical cell layer, next to endodermis, were able to absorb NR and plasmolyse and when stained with AO this layer was the only one that consistently had detectable nuclei. By the fifteenth day only a few cells in the inner cortical layer had these properties.

There was close agreement between the assessments of cell viability in wheat roots with AO and NR-plasmolysis, although the NR-plasmolysis method giving a slightly larger estimate of dead

Table 3.1 Numbers* of living cortical cell layers (max 6 for wheat, 5 for tomato) outside of the endodermis in sterile detached root pieces incubated for 0-15 days on mineral nutrient agar; assessment based on staining with acridine orange (AO), neutral red-plasmolysis (NR) or fluorescein diacetate (FDA).

Incubation of segments (days)	Wheat			Tomato		
	AO	NR	FDA	AO	NR	FDA
0	5.98±0.02 (5.90±0.05)	5.95±0.05	6.00±0.00	5.00±0.00 (5.00±0.00)	5.00±0.00	4.84±0.03
5	3.34±0.23 (3.14±0.09)	3.24±0.16	alive**	5.00±0.00 (4.95±0.03)	4.42±0.21	Alive
10	1.52±0.17 (1.39±0.17)	1.00±0.28	mainly dead	4.94±0.04 (4.86±0.07)	1.01±0.86	Mainly dead/ Dead
15	0.53±0.10 (0.35±0.09)	0.33±0.07	mainly dead/ dead	5.00±0.00 (5.00±0.00)	0.00	Dead

* Mean ± S.E. of mean for 5 root pieces, averaged along the lengths of root pieces, or 10 pieces (in parentheses) for roots stained with AO after previous staining with NR or FDA.

** Quantitative assessment not possible: "alive", most cells fluorescing; "mainly dead", fluorescence seen only deep in the cortex, perhaps originating from the stelar tissues; "dead", no fluorescence.

layers at all times. Comparing the assessments for the same parts of individual root pieces (microscope fields), Fig.3.1. shows a very highly significant correlation ($P<0.001$). These results confirm previous reports of comparisons between the two techniques for cereal roots (Holden, 1975; Henry & Deacon, 1981).

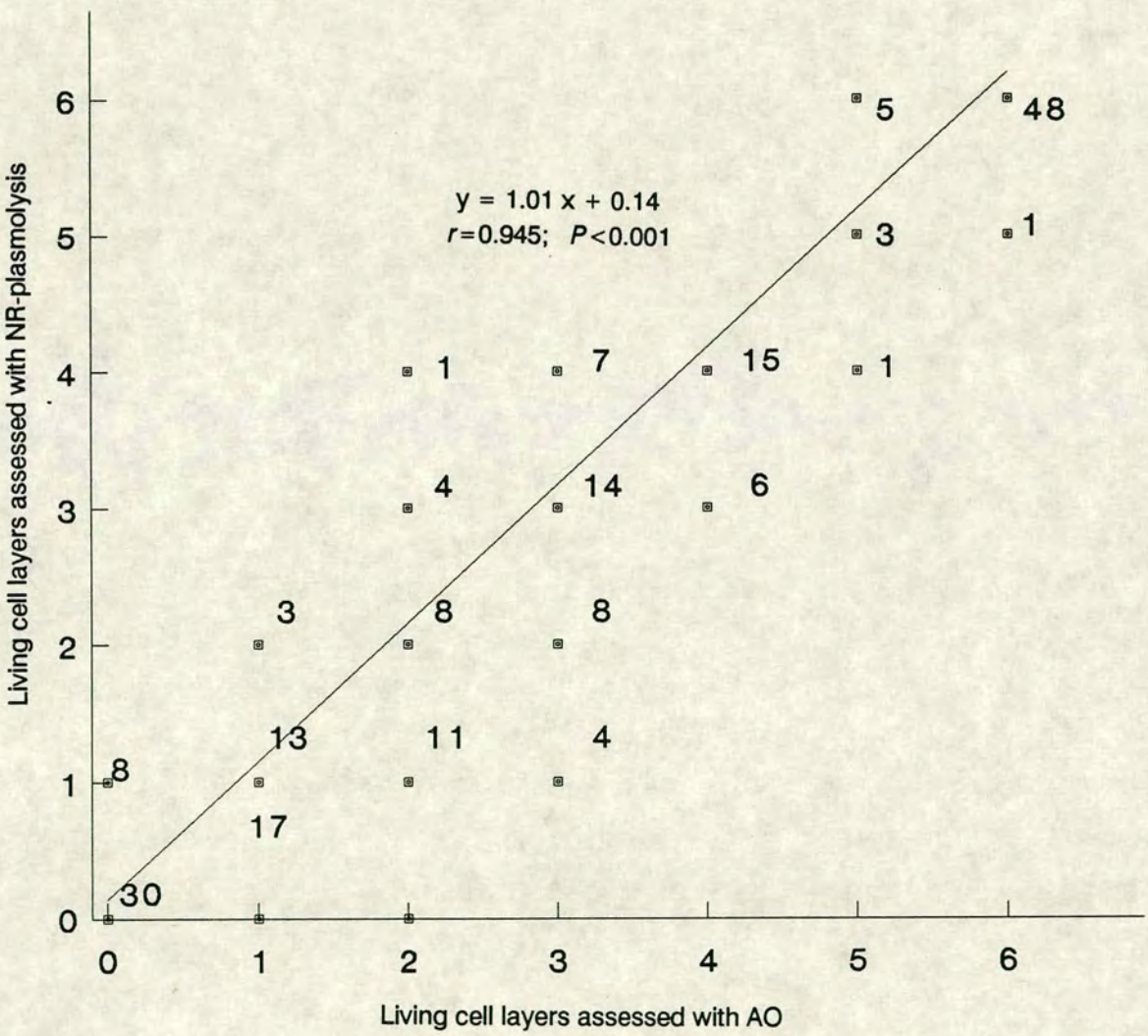
Comparison of AO and NR-plasmolysis in tomato root pieces

The cells of the root cortex in tomato were smaller than those of wheat and their nuclei were also smaller and more numerous per microscope field. They were assessed as being alive at day 0, but by the fifth day of incubation, some root pieces showed a loss of viability in cells of the outer cortical layers when assessed with the NR-plasmolysis method. The degree of cell death was significantly ($P=0.02$) less than that in wheat roots.

By day 10 there was considerable death of the tomato root cortex when assessed with NR-plasmolysis, but individual root pieces differed in this respect, reflected in the large standard error in Table 3.1. Some root pieces had no living cortical cells whereas others were fully alive. By the fifteenth day no living cell was detected with the NR-plasmolysis method. However, all cortical cells had stainable nuclei (AO, Fig. 3.3), although their fluorescence was not as bright as in the freshly excised root pieces. In tomato, as in wheat, the process of cell death was seen to begin in the outer layers of the cortex and to progress inwards with time, but this pattern was less definite than in wheat and its rate once it started was faster, suggesting a collapse of the root function rather than a programmed sequential senescence and death.

The most notable feature of the results from tomato was that the cortical cells retained nuclei that were detectable with AO at all

Figure 3.1. Relationship between assessments of living cortical cell layers (max.6) of detached wheat root pieces using acridine orange (AO) and neutral red staining followed by plasmolysis (NR-PI).*



* Each data point represents an assessment of the same point on a root assessed by the two methods (numbers of coinciding points are shown by numerals); results based on individual microscope fields of view of root pieces incubated for 0-15 days on mineral nutrient agar.

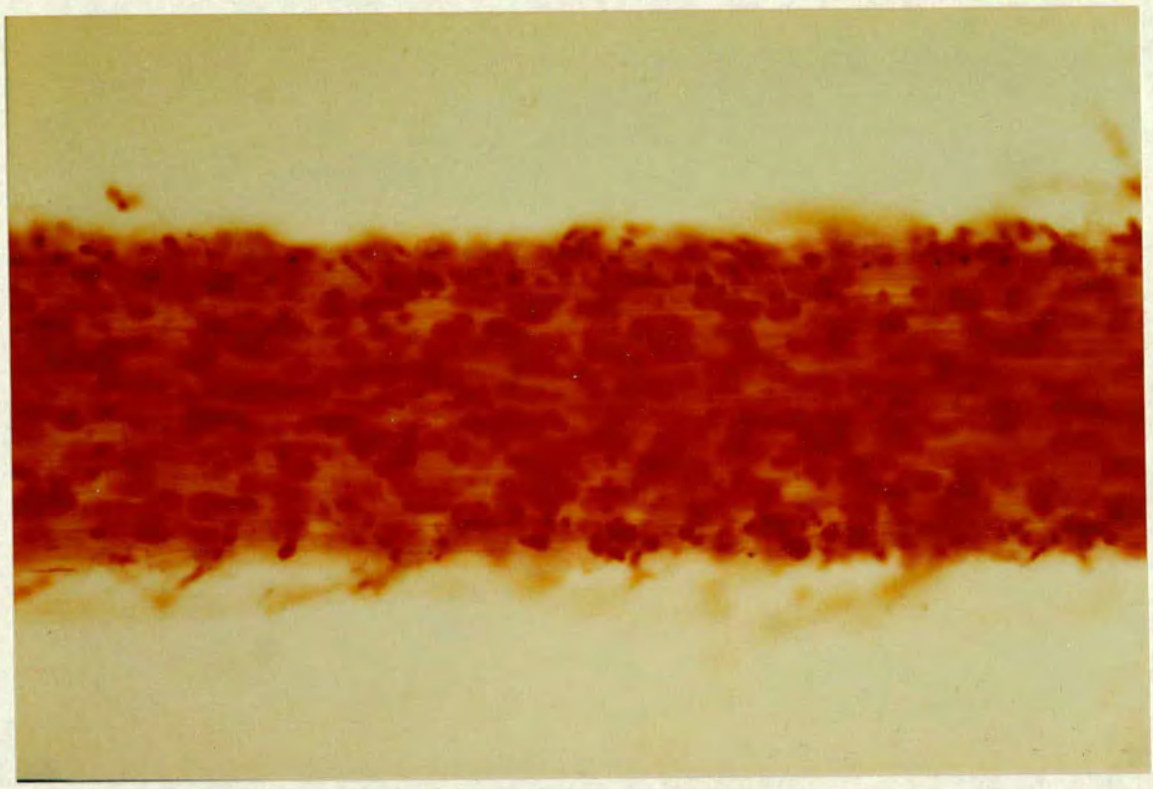


Fig. 3.2. NR-PI staining of tomato root; fully living cortex.

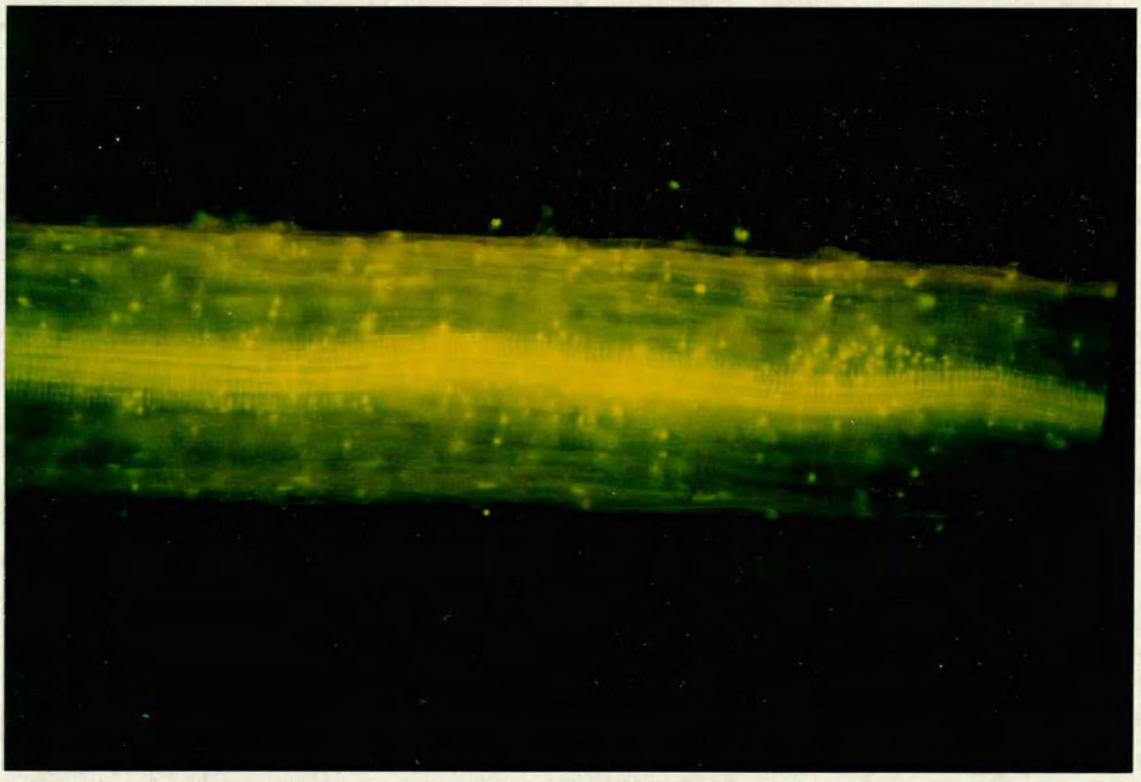


Fig.3.3. Tomato root with a dead cortex that did not plasmolyse when treated with NR but still retained nuclei stainable with AO.

incubation times, even though the cells failed to absorb NR and to plasmolyse and they showed obvious signs of degeneration. These results show that the assessment of root viability with AO, although valid for wheat, is not valid for tomato roots.

Evaluation of staining with FDA for assessment of viability of the root cortex of tomato and wheat

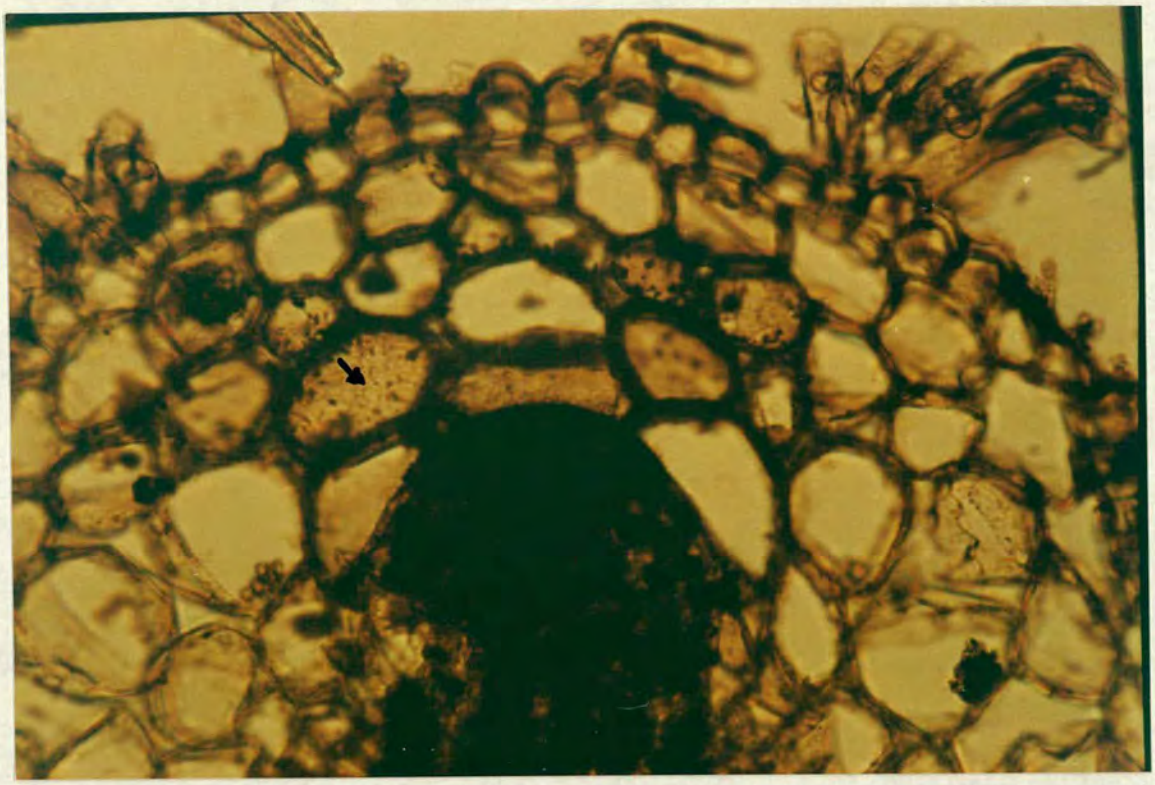
The use of FDA gave very poor results. Living cells did fluoresce and could be distinguished initially from adjacent dead cells but the fluorescence soon diffused from the living cells into the adjacent cells, at which stage it had no diagnostic value. Also, the fluorescence of cells deep in the tissues was of low intensity and not easily detectable unless a strip of the overlying dead cortex was removed. Moreover, the fluorescence decreased after the slide had been irradiated for some minutes with a UV beam and this resulted in loss of intensity of fluorescence in microscope fields adjacent to those being assessed at any one time. As a result of these problems it was impractical to obtain quantitative assessments of cell viability with FDA. However, as shown in Table 3.1, the observations on roots treated with this compound were broadly compatible with the assessments of cortical viability based on the methods of NR-plasmolysis and AO.

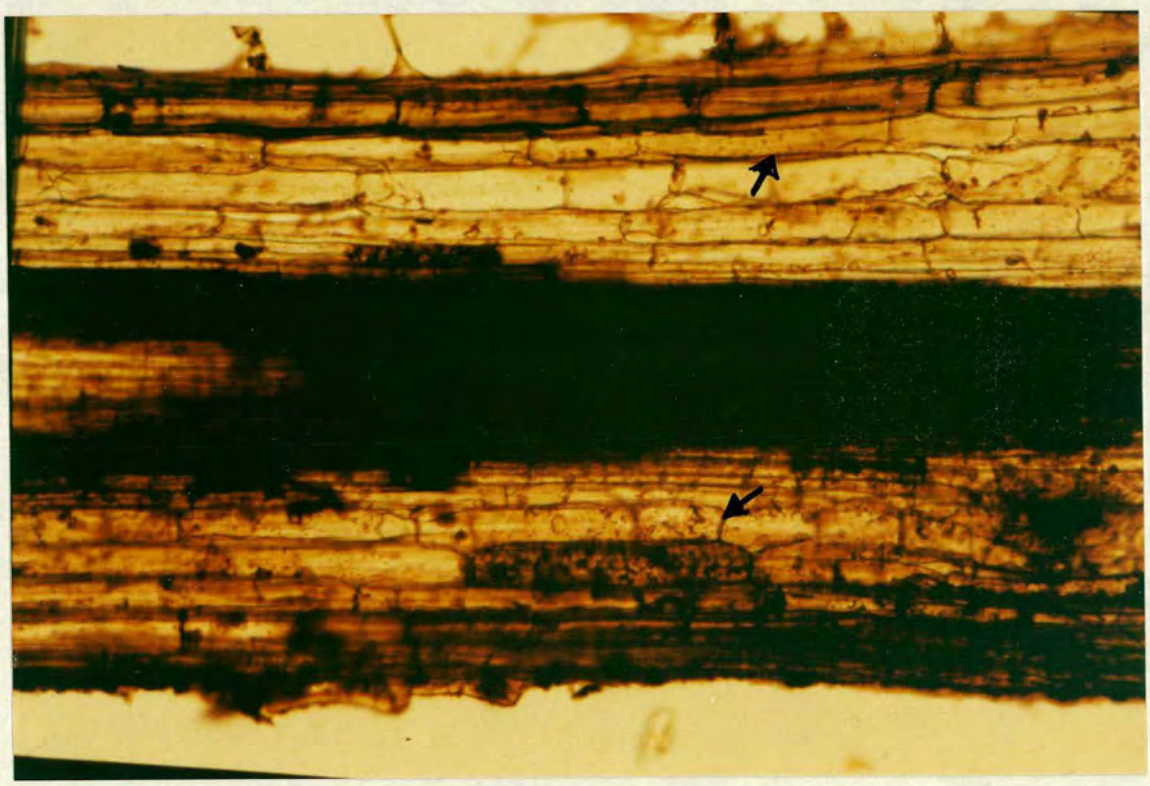
Cytochemistry of acid phosphatases and of DNAases

The method of Gomori (1952) as modified by Gahan (1965) for the acid phosphatases and of Daoust & Amano (1961) for DNAases were used to detect enzyme activities in freshly excised root pieces or after 5 days incubation on mineral nutrient agar (MA). For the assessment of acid phosphatases, the roots were sectioned with a freezing microtome; *p*-nitrophenyl

phosphate or β-glycerophosphate were used as substrates. Free-hand sections were used for the cytochemical test of DNAases.

The high degree of vacuolation of the fully differentiated cortical cells of both wheat and tomato roots did not permit very accurate localisation of enzymes in the cell cytoplasm. Some individual cells had rich cytoplasmic contents while other cells were empty of cytoplasm, either because they had been sectioned in highly vacuolated regions or because the cytoplasm had disintegrated during senescence. Staining reactions indicating acid phosphatase activities were found in the cell walls of both wheat and tomato roots. In sections of wheat roots some cortical cells showed particulate distribution of acid phosphatase activity (Figures 3.4, 3.5), presumably reflecting subcellular localization of activity in the living cytoplasm, but other cells showed a diffuse activity, perhaps reflecting cell senescence. In sections from freshly excised roots there were more cells with well preserved cytoplasm and both patterns of enzyme distribution were seen. But in sections from root pieces incubated for 5 days on MA, most of the cortical cells in the sections were "empty" and only the cell wall showed intense enzyme activity, especially in the epidermal cells. The high percentage of cells without cytoplasm precluded quantitative assessment of the distribution of cells showing different enzyme activities. Of interest, however, an increased incidence of cells with a particulate distribution of acid phosphatase activity was seen in the cortex of the root axes adjacent to lateral root primordia (Fig.3.4) as found by Sutcliffe & Sexton (1969) for pea roots that contained lateral root primordia. The particulate appearance of this activity suggests that the cells were alive (Gahan, 1966). In this respect, Henry & Deacon (1981) found, using AO staining, that nuclei





Figures. 3.4. & 3.5. Acid-phosphatase activity in root of wheat.

3.4. Transverse section, showing general staining reactions of cell walls and evidence of intracellular localisation of activity in cortical cells overlying root lateral initial (arrow) that is growing through the cortex.

3.5. Longitudinal section, showing a few individual cortical cells with evident sub-cellular localisation of acid-phosphatase activity (arrows).

persisted in cortical cells of root axes around the emerging laterals of cereal roots. In tomato root sections there were also cells with particulate appearance of the enzyme activity, but in most cases the intense staining reaction of the cell walls obscured the detailed distribution of enzyme activity. In contrast to wheat roots, tomato roots did not show differences in enzyme distribution or activity between freshly excised roots and those incubated on MA for 5 days.

DNAases did not show a clear difference in pattern of activity between cortices of freshly excised roots and 5 day old root pieces of wheat and tomato. There were, however, differences in DNAase activities between different parts of roots, more activity being seen in the stelar area than in the cortex. This reflects the inadequacy of the method used, which can detect differences mainly at tissue level rather than cellular level. In some instances, however, good sections of freshly excised wheat roots, 5-6 cm behind the tip, did show differences in DNAase activity between individual cells (Fig.3.6).

From all the results of this experiment it was found that cortical cell death in cereals could accurately be assessed by either the AO or NR-plasmolysis method, whereas cell death in tomato could be assessed only by NR-plasmolysis. On this basis, these methods (as appropriate) were used in all further experiments.

3.3. Effects of sugar supplements on cortical cell death in tomato root pieces

The rapid senescence of the cortex of tomato root pieces described in Section 3.2 might have been caused by starvation rather than a progressive programmed senescence as reported for cereal

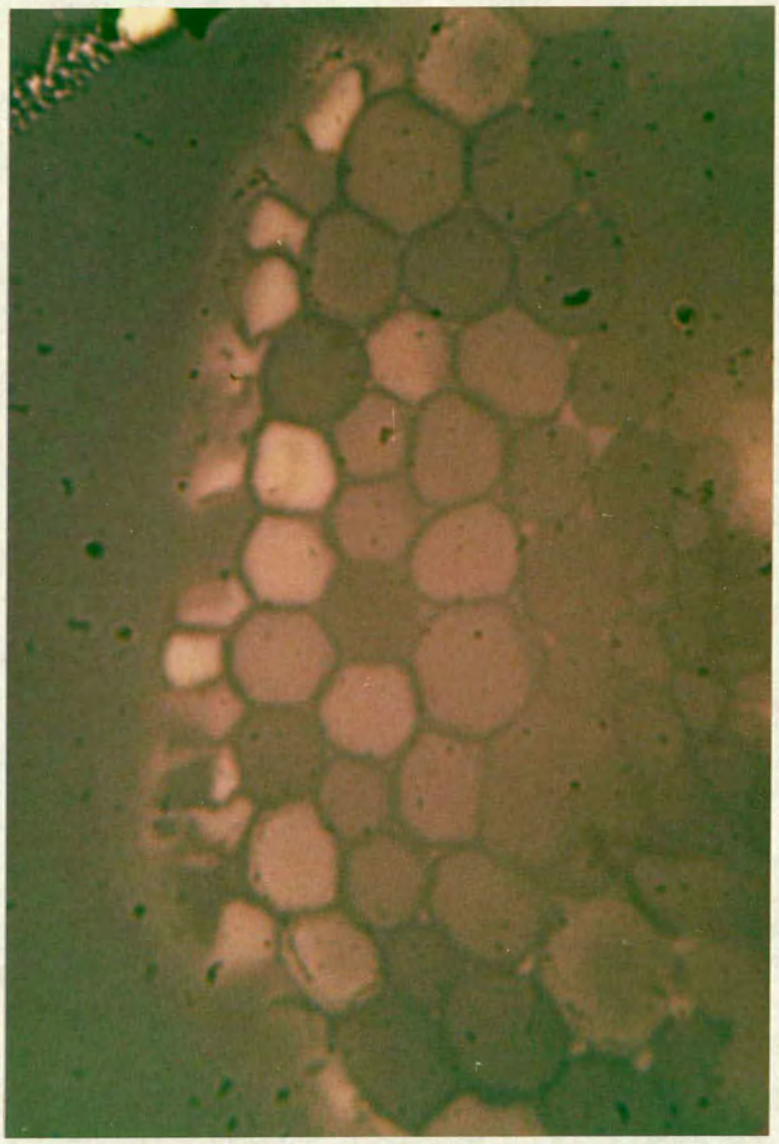


Fig. 3.6. DNAse activity in cortical cells of wheat. Transverse section of wheat root attached to the seedling, 5 cm behind the growing tip showing evidence of increased enzyme activity (light-colored cells) in the epidermis and hypodermis.

roots. To investigate this, tomato root pieces as in the previous experiment were incubated on agar media containing the minerals of White's medium and various concentrations of sucrose (0, 0.1, 0.5, 1 and 2%). After incubation for 10 or 15 days the root pieces were assessed for viability of their cortical cells, by the NR-plasmolysis method. Because there was no clear pattern of progression of cell death in the cortex of tomato, each cortical cell layer was taken to represent 20% of the volume of the cortex and the proportion of cell death in each layer was then assessed. So, for example, if half of the root epidermis was non-viable in one microscope field the cortex was scored as showing 10% death in that field.

3.3.1. Results

As shown in Table 3.2 much of the root cortex had died by 10 or 15 days in root pieces incubated on either water agar or mineral nutrient agar in the absence of sugar. In contrast, almost all of the cortex was alive after 10 days in the presence of even low levels of sucrose. Much also was alive after 15 days, but at this time there was an indication (not significant) that the highest level of sucrose (2%) maintained the viability of the cortex better than did the lowest glucose level (0.1%). Many laterals had developed from the root pieces in the presence of sucrose, although not in the absence of sucrose (Table 3.3). Their numbers increased progressively with sucrose concentration, although their mean length was less affected by sugar concentration. From these results it is clear that death in tomato root pieces is caused primarily by starvation. The process of death is different from that observed in the root cortex of cereals, where there is an ordered and sequential

Table 3.2 Percentage of the root cortex of detached tomato root pieces that was alive after 10 or 15 days on water agar (WA), mineral nutrient agar (MA), or mineral nutrient agar with different sucrose concentrations (MA+S).*

Incubation (days)	Composition of the medium					
	WA	MA	MA+0.1% S	MA+0.5% S	MA+1% S	MA+2% S
10	15±6 (16)	17±8 (19)	96±2 (10)	96±4 (10)	99±1 (10)	99±1 (10)
15	2±2 (14)	0 (17)	69±6 (8)	82±4 (8)	82±8 (8)	87±6 (6)

* Means with S.E. of mean for numbers of root pieces in parentheses

Table 3.3 Mean number* and length of laterals formed by detached tomato root pieces after 10 or 15 days incubation on mineral agar supplemented with different concentrations of sucrose.

	% SUCROSE				
	0	0.1	0.5	1	2
Mean number of laterals per root piece at 10 days	0	0.1±0.1 (20)	2.7±0.3 (18)	4.0±0.5 (20)	4.7±0.5 (19)
Mean length (mm) of laterals at 10 days	0	5.0±2.0 (2)	6.8±0.5 (50)	8.0±0.6 (72)	5.3±0.4 (90)
Mean length (mm) of laterals at 15 days	0	--	9.4±0.5 (28)	9.6±1.0 (36)	5.5±0.7 (44)

* Means ± S.E. of mean. In parentheses, total numbers of root pieces (first row), or laterals (second and third rows).

death of cortical layers from the outer cortex inwards, rather than total collapse of viability in large areas of the root as occurs in tomato in the absence of nutrients. It is noteworthy that concentrations of sucrose as low as 0.1% were sufficient to maintain viability of the tomato root cortex over a relatively long time.

3.4. Effect of excision of seed, shoot or root tip on RCD of wheat

This experiment was designed to investigate the influence of different parts of the germinating seedling on RCD. In a factorial experiment the effects of removal of the seed, shoot and root tip on RCD of the seminal roots were studied.

Sterile 5 day old wheat seedlings were prepared in dishes with mineral nutrient agar, as described in Section 2.4. All roots except the first-formed seminal root were removed and then the shoot, seed, or root tip was removed with a sterile scalpel and forceps so that all eight combinations of excision (or not) of the three parts of seedlings were tested. The shoot was cut at its base and the root at 5 mm behind the tip. The seed was removed by pulling it gently from the rest of the seedling (this was facilitated by holding the base of the shoot with forceps) so that the seed-coat and its contents were removed, leaving only the scutellum attached to the seedling. The 20 mm region of root, starting 5 mm behind the tip, was marked on the Petri dish in each case so that samples of root pieces of uniform origin and age were sampled at the end of the experiment. The roots were sampled after 4 and 6 days' incubation and sampling time also was included as a factor in analysis of variance of the experiment. There were 9 replicate root pieces for each treatment at

each sampling time. Assessments were made along the length of root pieces stained with AO, the numbers of nucleate cortical layers in successive microscope fields being recorded.

3.4.1 Results

The mean numbers of nucleate cortical layers for each combination of excision treatment (pooled together for both sampling times) are shown in Table 3.4. Analysis of variance of the data shows that removal of any part of the seedling shoot, seed or root tip caused a decrease in RCD and this was highly significant for the removal of the shoot or the root tip but not significant for seed removal.

There was no interaction between the three "removal" factors but there was a significant interaction (P=0.001) between the factors "seed removal" and "day of sampling", caused by a delayed RCD at the 4-day sampling and an increased RCD at the 6-day sampling in treatments with seed attached, compared with seed detached. A summary of this effect (factors "shoot" and "root tip" are pooled together) is shown in Table 3.5.

The roots produced many laterals, especially when the seed was attached and the root tip and shoot were removed. When the seed was detached lateral primordia also were seen but they had not grown. The persisting nuclei in cells around these laterals were not taken into account when the roots were assessed for RCD. There was no correlation between number of laterals (or lateral primordia) and RCD in each root piece assessed (r=0.122)

Table 3.4 Number of nucleate cortical cell layers in 2 cm lengths of wheat root from seedlings whose seed, shoot or root tip was removed.*

Shoot	Seed	Root tip		Row mean
		Attached	Removed	
Attached	Attached	2.7	3.3	*3.0*
	Detached	3.1	3.4	*3.2*
Detached	Attached	3.5	3.7	*3.6*
	Detached	3.2	4.2	*3.7*
Column mean		**3.1**	**3.6**	

	S.E.D.	L.S.D.(1%)
* Comparison of any two single means	0.211	0.6
Comparison of any two "shoot X seed" means (italicised)	0.149	0.4
Comparison of the two "Root tip" means (bold)	0.106	0.3

Table 3.5 Effect of seed removal from wheat seedlings on the number of nucleate cell layers in their roots, after 4 and 6 days.*

Day of sampling	Seed attached	Seed detached	Row mean
4	4.1	3.9	4.0
6	2.4	3.0	2.7
Column mean	3.3	3.5	

	S.E.D.	L.S.D.(1%)
* Comparison of any two single means	0.149	0.4
Comparison of column or row means.	0.106	0.3

3.5. Effect of removal of the root tips, presence of mineral nutrients and presence of sucrose on cortical senescence in wheat root pieces

In this experiment, the effect of mineral nutrients and of sucrose in combination with presence or absence of the root tip was studied in sterile 3 cm long wheat root pieces. The experiment was designed as a factorial with four factors each at two "levels" : presence/absence of minerals, presence/absence of 2% sucrose, tip removed or attached, and length of incubation (4 and 6 days). All combinations of treatments were used for each sampling time, with 7 replicate root pieces. Roots were assessed for RCD with AO along their original length (any new growth being excluded).

Because in previous experiments a progressive increase in numbers of nucleate cell layers was seen from the basal end of the root piece towards the apical end, RCD of each quarter of the length in each root piece was assessed and analysed separately.

3.5.1. Results

Table 3.6 shows the mean number of nucleate cell layers in each part of the root pieces. Part A is the first quarter starting from the basal end, and part D is the apical quarter.

As was expected, the 6 day sampling always revealed more RCD than at the 4-day sampling, and sampling time did not interact with the other factors so the results were pooled for both sampling times.

The root pieces had a progressively increasing number of nucleate layers from the basal part towards the apical part ($P<0.001$) irrespective of treatment. In the experiment overall, the presence of minerals decreased slightly the amount of RCD in the younger parts of root pieces (significant for parts B and

Table 3.6 Mean number of nucleate cortical cell layers along excised wheat root pieces incubated in medium amended or not amended with sucrose or mineral nutrients. Results pooled for two incubation times, 4 and 6 days.

Presence (+) or absence(0) of 2% Sucrose	Mineral nutrients	Part of root piece* A	B	C	D	Row mean	New growth from tip (mm)
			Root tip attached				
+	+	1.8	2.4	2.8	4.2	2.8	17.1±1.4
+	0	2.0	1.7	2.3	3.5	2.4	24.0±2.2
0	+	2.4	2.4	3.0	3.9	2.9	2.1±0.1
0	0	2.0	2.0	2.6	4.3	2.7	0.8±0.3
			Root tip detached				
+	+	2.3	3.4	3.8	5.0	3.6	n.a.
+	0	3.6	2.9	3.0	4.8	3.6	n.a.
0	+	2.0	1.9	2.5	4.2	2.7	n.a.
0	0	2.5	1.9	2.3	3.6	2.6	n.a.
Column mean		2.3	2.3	2.8	4.2		
S.E.D.		0.41	0.31	0.37	0.31	0.25	
L.S.D.(1%)		1.1	0.8	1.0	0.9	0.7	

* A, first quarter of root piece starting from the base; B, second quarter; C, third quarter; D forth quarter (nearest the tip). Means of 18 replicates.
n.a, not applicable.

C, P=0.05), but not in the older part. Overall, sucrose had no significant effect on RCD, nor did excision of the root tip. But there was a very strong statistical interaction between these treatments (P=0.05 for region A, P<0.001 for region B, P=0.002 for region C, P<0.001 for region D). There was an increase in numbers of nucleate cell layers when the root tip had been removed and sugar was present in the incubation medium and this was constantly observed in all four regions along the root pieces. In contrast, in the absence of sucrose there was no significant effect of removal of the root tips.

3.6. RCD of wheat root pieces incubated in split agar plates

In this experiment Petri dishes (9cm diam.) with a central partition were used, the two halves of each dish being filled with various combinations of agar media. Two depressions were made in the central partition and 3 cm long root pieces were placed across the depressions so that they bridged the two media but there was no direct contact between the media. An agar block, 1x1x0.5 cm, cut from the appropriate medium, was placed on top of each end of the root piece to ensure good contact of the cut ends with the medium. (Fig.3.7). The media used were: White's minerals plus 2% sucrose agar (MSA), White's minerals agar (MA) and water agar (WA). All combinations of the three media were used between base and tip of the root piece, giving 9 combinations in all. The combinations are denoted as follows: MSA → WA where the first symbol is the medium supporting the base of the root and the last symbol is that supporting the tip.

The root pieces were incubated for 5 and 7 days and then assessed for RCD with the AO method in all microscope fields along the root piece. There were 5 replicate root pieces for each

treatment at each time. In order to enable detection of differences along the lengths of the root pieces, the average number of nucleate cortical layers was estimated separately for each quarter of the root length, starting from the base (part A) towards the tip (part D) of the original parts of the root. Any new growth was assessed separately. The analysis of variance of the results was done separately for each region of the root pieces.

The experiment was done twice, first using root pieces with tips and then using root pieces with the tips removed. In the second experiment any laterals that developed during incubation were excised with a sterile scalpel within 1 day of their appearance.

3.6.1 Results

Tables 3.7 and 3.8 show the mean numbers of nucleate cortical cell layers in root pieces with tips attached and detached respectively, the data being pooled for the 5- and 7-day samplings because these showed the same pattern of results. Because the experiments were done at different times, the data for attached and detached roots cannot be directly compared.

In both experiments there was a highly significant difference ($P<0.001$) in RCD between the parts of the root pieces. The "apical" parts always had the least RCD irrespective of the media used. In the experiment where the tips were removed there was always a progressive increase in RCD from the apical to the basal end of root pieces for all media combinations. When the tip was present, however, there was a tendency for nuclei to persist in the "basal" quarter of the root pieces more than in the central region; this

effect was significant (P=0.02) and consistent for all combinations of media. In other words, in both experiments the pattern of RCD along the root pieces was not influenced by the combination of the media.

There were, however, significant effects of the media on the rate of RCD. For example, when the root tips were attached and the basal parts of root pieces were on sugar-free agar, the presence of sugar in agar supporting the apical regions caused a significant reduction in RCD overall. This was not true, however, in the experiment where the root tips were excised - compare for example, the data for WA → MSA and WA → WA in Table 3.8. But in both experiments if the basal regions were on mineral agar then the presence of sucrose in agar supporting the apical regions resulted in a significant reduction of RCD overall.

The media combinations MA→MSA and MSA→MA showed the least RCD (averaged for the four root regions) in both experiments and this was reflected in the least RCD in all parts of root pieces except the basal regions.

There were other statistically significant differences between the various media combinations in the two experiments, but in all cases the effect was relatively small. It was not, for example, possible to maintain full viability of any part of a root with any combination of nutrients, as had been hoped when the experiment was designed.

A final notable feature (Table 3.7) was the effect of sucrose in promoting root tip growth. In this respect, conspicuous further growth of root tips occurred if sucrose was applied to either the

Table 3.7 Number of nucleate cortical cell layers along excised wheat root pieces incubated in split plates containing different combinations of media. Results pooled for both incubation times (5 and 7 days); tips not removed.

Medium combination***	Part of root piece * A	B	C	D	Row mean	Length of new growth (mm)**
MSA → MSA	3.0	2.6	2.3	4.6	3.1	26.6±1.9
MSA → MA	2.8	2.7	3.6	5.2	3.5	8.1±0.6
MSA → WA	3.4	2.6	2.6	4.6	3.3	11.4±1.3
MA → MSA	3.9	3.5	3.3	4.6	3.8	23.9±2.3
MA → MA	2.7	2.3	2.8	4.7	3.1	1.1±0.2
MA → WA	2.5	2.2	2.6	4.4	2.9	2.1±0.4
WA → MSA	3.2	2.5	3.2	5.1	3.5	20.9±2.8
WA → MA	2.0	1.9	2.6	4.9	2.8	3.2±0.4
WA → WA	2.1	1.5	1.7	4.2	2.4	2.0±0.4
S.E.D.	0.57	0.40	0.44	0.34	0.22	
L.S.D.(1%)	1.5	1.1	1.2	0.9	0.6	
Column mean		2.9	2.4	2.7	4.7	S.E.D. 0.15 L.S.D.(1%) 0.4

* A First quarter of the root segment starting from the base, B second quarter, C third quarter, D forth quarter (tip); means of 10 replicates.

** Measured the 5th day for all root pieces.

*** MSA, Minerals + Sucrose agar; MA, Mineral agar; WA, water agar.

Table 3.8 Average number of nucleate cortical cell layers along excised wheat root pieces incubated in split plates containing different combinations of media. Results pooled for both incubation times (5 and 7 days); tips removed.

Medium combination**	Part of root piece* A	B	C	D	Row mean
MSA → MSA	1.0	1.6	2.5	4.2	2.3
MSA → MA	1.0	3.0	4.0	4.8	3.2
MSA → WA	1.5	1.8	1.8	3.4	2.1
MA → MSA	0.5	3.7	3.6	4.7	3.1
MA → MA	1.0	1.7	2.3	3.6	2.2
MA → WA	1.3	2.1	2.6	3.9	2.5
WA → MSA	1.3	1.6	1.1	2.6	1.7
WA → MA	0.5	1.6	2.3	3.5	2.0
WA → WA	0.5	1.6	2.4	3.7	2.0
S.E.D.	0.41	0.45	0.51	0.51	0.24
L.S.D. (1%)	1.1	1.2	1.4	1.4	0.6
Column mean	1.0	2.1	2.5	3.8	S.E.D. 0.16 L.S.D. (1%) 0.41

* A, first quarter of the root segment starting from the base; B, second quarter; C, third quarter; D forth quarter (tip); means of 10 replicates.

** MSA, Minerals + Sucrose agar; MA, Mineral agar; WA, water agar.

basal or the apical part of root pieces. But by far the largest amount of root tip growth occurred when sucrose was applied to the apical regions.

3.7. Effects of plant growth regulators on RCD

Growth regulators play an important role in various aspects of physiology. Of all the known plant hormones, cytokinins and ethylene are those most closely involved in plant senescence. In an initial attempt to investigate such effects on RCD, root pieces with tips attached or detached were incubated on mineral agar amended with indolyl acetic acid (IAA), gibberelic acid (GA_3) or benzylaminopurine (BAP), at concentrations of 10^{-4}, 10^{-5} and 10^{-6} M each. There were also two sampling times (7 replicates for each combination of tip removal, sampling time, and incubation medium).

Because it is known that cytokinins inhibit the formation of laterals in excised root pieces incubated on sugar-containing media (Gillespie, 1986), it is possible to exert an indirect effect on RCD by stopping the formation of laterals, a powerful sink of plant resources. So another experiment was done in which benzylaminopurine at concentrations of 10^{-5} and 10^{-6} M was used in mineral agar amended with 2% sucrose.

3.7.1. Results

Table 3.9. shows the numbers of nucleate cell layers along wheat root pieces in the presence of the different chemicals, the data being pooled for two sampling times and root pieces with tips attached or detached. IAA decreased the amount of RCD in all but the youngest root region at concentrations of 10^{-6} and 10^{-5} M, while at a concentration of 10^{-4} M it accelerated cortical death in the older

Table 3.9 Number of nucleate cortical cell layers along excised wheat root pieces incubated on medium containing plant hormones at various concentrations. Results pooled for two incubation times, 4 and 6 days, and for root pieces with tips attached or removed.

Treatment*	Part of root piece** A	B	C	D	Row mean
Control	3.1	3.0	3.4	3.9	3.4
IAA 10^{-6} M	4.1	3.9	4.0	4.0	4.0
IAA 10^{-5} M	4.5	4.6	4.5	4.3	4.5
IAA 10^{-4} M	1.8	2.9	3.3	4.2	3.0
GA_3 10^{-6} M	2.2	2.2	2.9	3.3	2.6
GA_3 10^{-5} M	1.8	1.7	1.8	2.5	1.9
GA_3 10^{-4} M	2.4	2.1	2.4	3.1	2.5
BAP 10^{-6} M	2.6	1.7	2.1	3.3	2.5
BAP 10^{-5} M	2.1	2.3	3.0	3.4	2.7
BAP 10^{-4} M	2.2	2.6	2.9	3.6	2.8
S.E.D.	0.33	0.27	0.29	0.27	0.29
L.S.D. (1%)	0.9	0.7	0.8	0.7	0.8
Column mean	2.7	2.7	3.0	3.6	S.E.D. 0.092
					L.S.D.(1%) 0.3

* IAA, indolylacetic acid; GA_3, gibberelic acid; BAP, benzylaminopurine.

** A, First quarter of the root piece starting from the base; B, second quarter; C, third quarter; D, fourth quarter (nearest to tip).
S.E.D., Standard error of difference between any two means of the same part of root piece (DF=155).

Table 3.10 Mean number of nucleate cortical cell layers along excised wheat root pieces incubated on medium containing 2% sucrose with or without benzylaminopurine. Results pooled for two incubation times, 4 and 6 days.

Treatment	Part of the root piece* A	B	C	D	Row mean
Control	2.2	2.5	2.8	3.0	2.6
BA 10^{-6} M	2.1	2.0	2.1	2.0	2.0
BA 10^{-5} M	2.4	2.3	2.5	3.0	2.5
S.E.D.	0.35	0.25	0.22	0.25	0.2
L.S.D. (1%)	0.9	0.7	0.6	0.7	0.7

* A, first quarter of the root piece starting from the base; B, second quarter; C, third quarter; D, fourth quarter (nearest to tip).

S.E.D., Standard error of difference between any two means of the same part of root piece (32 replicates).

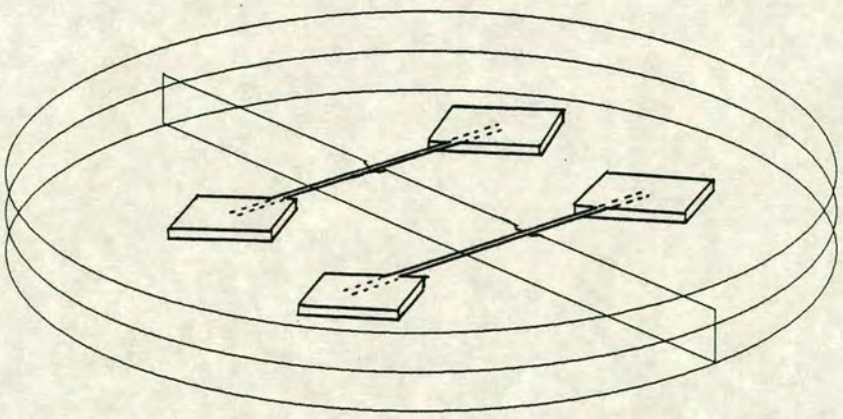


Fig.3.7. Split agar plate with central partition.

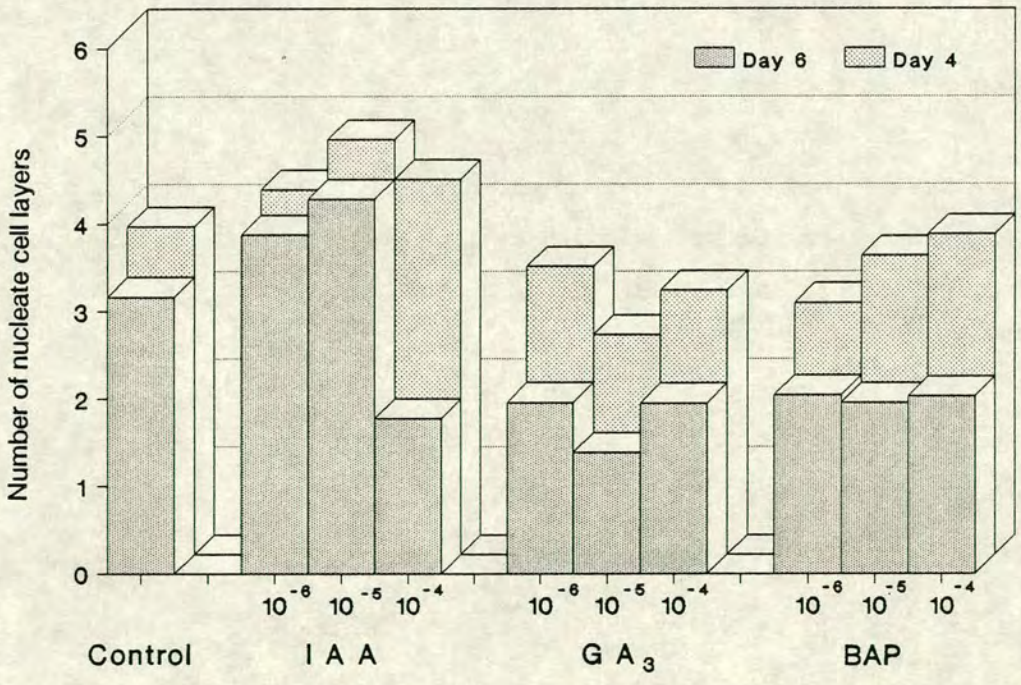


Figure 3.8. Number of nucleate cortical cell layers of wheat root pieces incubated for 4 and 6 days on agar amended with indolylacetic acid (IAA), gibberellic acid (GA_3) or benzylaminopurine (BAP).

parts of the root pieces. Gibberelic acid and benzylaminopurine increased RCD at all concentrations used.

Analysis of variance of the results in this experiment showed a strong interaction between the factors 'incubation medium' and sampling time. This effect is shown in Fig. 3.8. GA and BAP had an effect in accelerating RCD after the 4th day while IAA at molar concentrations 10^{-5} and 10^{-6} M delayed RCD but it accelerated RCD at concentration 10^{-4} M.

Table 3.10. shows the numbers of nucleate cell layers of wheat root pieces incubated on mineral nutrient agar amended with 2% sucrose and BAP at concentrations of 10^{-5} and 10^{-6} M. The root pieces in the control produced numerous laterals but not those amended with BAP at either concentration. BAP at 10^{-5} M had no effect on RCD, but at 10^{-6} M it significantly enhanced RCD in the younger root regions. This contrasted with the results in the absence of sucrose, where BAP significantly increased RCD only in the older parts of root pieces.

3.8. Effect of Ag^{+} and of Co^{2+} ions on RCD of excised wheat roots

As an extension of the previous experiments, the effects of Ag^{+} and Co^{2+} on RCD were assessed. The cobalt ion is an inhibitor of ethylene biosynthesis (Yu and Yang, 1979), and Ag^{+} is an ethylene antagonist (Lieberman, 1979; Aharoni *et al.*, 1979). When either sprayed onto fruit or infiltrated into fruit at non-toxic concentrations prior to harvest, these ions delayed fruit ripening. Removal of ethylene also results in delay of ripening and senescence of fruits and flowers (Dilley, 1977).

To test the effects of these ions on RCD, $AgNO_3$ (at concentrations 0.05, 0.2, 1 and 5 μM) and $CoCl_2$ (at concentrations 10

and 50 μM) were added to water-agar which was used instead of mineral nutrient agar as the supporting medium for root pieces in order to avoid any precipitation of the ions.

3.8.1. Results

Table 3.11 shows the results of this experiment, pooled for root pieces with and without their tips. Both ions had an effect in reducing RCD after 4 and 6 days, compared to the controls. The differences were small, equivalent to about one cortical cell layer, but the numbers of nucleate cell layers were constantly increased for both ions in almost all parts of the root pieces; they were statistically significant for the younger parts of root pieces (P=0.002, <0.001 and <0.05 for parts B, C, and D respectively). These results suggest that ethylene may play a role in the process of senescence of cortical cells.

3.9. Effect of free radical scavengers, ascorbic acid and cycloheximide on RCD of wheat root pieces

This experiment was designed to investigate the effects of sodium benzoate and gallic acid which are considered as scavengers of free radicals. Because it was not known at which concentrations these chemicals might be active as scavengers or perhaps toxic to the roots, they were examined at the concentrations 0.25, 0.5, 1, 2, and 5 mM. Ascorbic acid, a reducing agent which may have an indirect effect on maintenance of cell membranes was also examined at concentration of 2 mM. Cycloheximide at low concentrations inhibits protein synthesis on 80S ribosomes and is readily taken up by cells. It has been found to inhibit senescence (chlorophyll and protein

Table 3.11 Mean number of nucleate cortical cell layers along excised wheat root pieces incubated on water agar containing silver or cobalt ions in various concentrations. Results pooled for two incubation times, 4 and 6 days, and for root pieces with attached and detached root tips.

Treatment	Part of root piece *				Row mean
	A	B	C	D	
Control	2.3	1.8	2.2	3.3	2.4
$AgNO_3$					
0.05 μM	2.6	2.4	2.7	3.3	2.7
0.2 μM	2.5	2.6	2.7	3.7	2.9
1 μM	3.0	2.2	2.6	3.0	2.7
5 μM	2.9	2.5	2.3	3.1	2.7
$CoCl_2$					
10 μM	2.5	2.4	2.9	3.4	2.8
20 μM	3.2	2.8	3.3	3.8	3.3
S.E.D.	0.31	0.22	0.22	0.24	0.25
L.S.D.(1%)	0.8	0.6	0.6	0.5	0.7

* A, first quarter of the root piece starting from the base; B, second quarter; C, third quarter; D, fourth quarter (nearest to tip).
S.E.D., Standard error of difference between any two means of the same part of root piece (28 replicates).

loss) in leaf disks (Frenkel *et al.*, 1968; Shibaoka & Thimann, 1970). It was used at concentrations of 1, 5, 10 and 20 µg/ml. In all cases these chemicals were incorporated into water agar on which root pieces were incubated.

3.9.1. Results

Table 3.12 shows the results of this experiment. The scavengers of free radicals, sodium benzoate and gallic acid, at the lower concentration (0.25 mM) had no significant effect on RCD. At higher concentrations they had a deleterious effect. But there was an anomalous result at the highest concentration of gallic acid, which was perhaps due to preservation of nuclei in dead cells.

Ascorbic acid seemed to change the pattern of nuclei loss in the cortical cells. There were differences in the numbers of nuclei between the two "sides" of each root piece, probably representing a difference between the side in contact with the agar medium and that furthest from it. It was also observed that nuclei persisted in some epidermal cells and root hairs. These unusual localised effects were not observed in any other experiment in this thesis and it is not certain if the nucleate cells were alive at sampling or if ascorbic acid just preserved the nuclei in dead cells.

Cycloheximide at 1, 5 and 10 mg/ml reduced RCD in some parts of the root pieces. At the highest concentration used, however, it seemed to be toxic.

Table 3.12 Mean number of nucleate cortical cell layers along excised wheat root pieces incubated on water agar containing various chemicals. Results pooled for two incubation times, 4 and 6 days, and for root pieces with tips attached or detached.

Treatment	Part of root piece *				Row mean
	A	B	C	D	
Control	2.1	2.0	2.1	2.9	2.3
Sodium benzoate					
0.25 mM	2.2	1.9	2.0	2.1	2.1
0.5 mM	2.1	1.8	1.5	1.7	1.8
1 mM	1.0	0.8	0.9	0.7	0.8
2 mM	0.4	0.5	0.4	0.3	0.4
5 mM	0.3	0.2	0.3	0.2	0.2
Gallic acid					
0.25 mM	2.0	1.4	1.8	2.4	1.9
0.5 mM	2.4	1.2	1.4	1.9	1.7
1 mM	0.9	0.6	0.7	1.0	0.7
2 mM	2.0	2.1	2.2	0.9	1.8
5 mM	2.6	2.8	2.9	2.8	2.8
Ascorbic acid					
2 mM	2.3	2.6	2.3	2.5	2.4
Cycloheximide					
1 µg/ml	2.6	3.0	2.9	3.2	2.9
5 µg/ml	2.5	2.2	2.2	3.4	2.6
10 µg/ml	3.0	3.3	2.8	1.4	2.6
20 µg/ml	0.6	1.4	1.7	0.5	1.0
S.E.D.	0.38	0.28	0.28	0.29	0.31
L.S.D.(1%)	1.0	0.8	0.8	0.7	0.8

* A, first quarter of the root segment starting from the base; B, second quarter; C, third quarter; D, forth quarter (nearest to tip).
S.E.D., Standard error of difference between any two means of the same part of root segment (32 replicates).

3.10. Invasion of pieces of sterile wheat root by *Microdochium bolleyi*

Excised root pieces of wheat were prepared as in Section 2.4 and incubated on White's mineral agar for 0, 2 and 4 days at 25°C in darkness, then they were placed tangentially along the margins of 6-day-old colonies of *M. bolleyi* on PDA, the margins being half-way across the PDA plates. There were 5 replicates for each treatment. Control root pieces were maintained on uninoculated PDA and on White's mineral agar, and were incubated in the same conditions for 1, 2, 3, 4, and 5 days. On sampling, all root pieces were assessed for cell viability by NR-plasmolysis. They were then decolorised in 70% methanol, stained with AO and assessed for numbers of nucleate cortical layers. Both methods were used in case challenge by the fungus caused loss of cell viability that was not detectable by AO staining.

Because the fluorescing fungal mycelium on the root surface and the dark chlamydospore-like structures of the fungus sometimes obscured AO fluorescence, the 5 root pieces from each sample were transversely sectioned (50 μm thickness) in a freezing microtome and the number of nuclei in each cell layer was counted in 28 sections taken at random from each sample, pooled across the replicates. Sections of the same root pieces were also stained with trypan blue to assess depth of fungal penetration. Fifteen sections taken at random from each treatment were used for this. Because the depth of fungal penetration was not uniform around the circumference of each root, each transverse section was divided arbitrary into six equal sectors and the deepest cell layer with fungal hyphae was recorded for each sector. The cortex of the wheat seminal root axis has usually six cortical layers, invasion of which was scored from

1 to 6; a score of 7 was given for invasion of the endodermis, 8 for the pericycle and 9 for the phloem and xylem. Two different modes of fungal penetration of the root cortex - intercellular and intracellular - were observed and recorded. Sections of the same roots were also stained with phloroglucinol-HCl and assessed for lignification of the cortical cells. Also, sections stored in 70% methanol were examined for autofluorescence under a UV beam (see Section 2.6).

3.10.1. Results

Table 3.13 shows assessment of viability of the cortical cell layers of wheat root pieces placed on colonies of *M. bolleyi* immediately after excision and incubated in the presence of the fungus. Also shown are assessments of freshly excised root pieces incubated on PDA or mineral agar for 0-5 days. Tables 3.14 and 3.15 show corresponding results for root pieces maintained on mineral nutrient agar for 2 and 4 days after excision before they were transferred to colonies of *M. bolleyi* or to PDA and mineral nutrient agar. Figures 3.9 - 3.11 show results equivalent to those in Tables 3.13 - 3.15 but based on assessments of nuclear distributions in root sections rather than in intact root pieces. Table 3.16 shows the depth of penetration of roots by *M. bolleyi* in all relevant treatments.

As the interpretation of some of these tables depends on the data in other tables, it is convenient to consider the findings in different ways, as follows.

3.10.1.1. Comparison of assessments based on AO and NR-plasmolysis.

Figures 3.15 - 3.18 show some representative wheat roots stained with NR. It was not always practicable to assess roots by both AO

and NR-plasmolysis, but in cases where this was done for the same root pieces (Tables 3.13 - 3.15) the NR method usually showed a slightly greater level of cell viability than did the AO method. The only exceptions were for roots incubated on mineral agar where sometimes the AO method showed slightly the more cell viability. Nevertheless, the differences between the assessment methods were small in all cases.

To compare the methods further, correlations were made, first between assessments of the numbers of living cortical cell layers, using AO stain and NR-plasmolysis of the same whole root pieces, and second between NR-plasmolysis assessments of whole root pieces and AO assessment of 50 μm transverse sections of the same lot of root pieces. Fig. 3.12 A shows this relationship for non-inoculated root pieces and 3.12 B for root pieces incubated on colonies of *M. bolleyi*. The data for these correlations were pooled across inoculation, pre-incubation and sampling times; for the sections a cell layer with less than 20% of nucleate cells was considered dead, as it is likely to appear so when viewed through the depth of an intact root. In all cases the slopes of the regression lines were near unity and the correlations were very highly significant. It seems therefore that any of these criteria of cell viability can validly be applied to roots that show natural progressive cortical senescence or cell death that may be enhanced by invasion by *M. bolleyi*.

3.10.1.2. Effects of time of pre-incubation on cortical cell death

Comparison of Tables 3.13 - 3.15 or Figures 3.9 - 3.11 shows that pre-incubation of root pieces on mineral agar before transfer to MA, PDA or colonies of *M. bolleyi* resulted in an increased degree

Table 3.13 Numbers of living cortical cell layers outside of the endodermis in sterile detached root pieces placed immediately after excision on colonies of *M. bolleyi* or on PDA or mineral nutrient agar, and sampled after 1-5 days; assessments based on staining with neutral red-plasmolysis (NR-Pl) or acridine orange (AO).*

Days of incubation	INCUBATION ON Colonies of *M. bolleyi*.		INCUBATION ON PDA		INCUBATION ON Min. Agar.	
	AO	NR-Pl	AO	NR-Pl	AO	NR-Pl
1	ND**	4.22±0.19	5.53±0.17	5.62±0.17	5.84±0.08	ND
2	ND	2.70±0.78	4.61±0.60	5.80±0.12	4.20±0.25	4.36±0.14
3	ND	2.12±0.53	4.01±0.53	4.22±0.45	3.82±0.07	ND
4	ND	1.36±0.56	1.16±0.75	1.72±1.10	2.70±0.29	ND
5	ND	2.17±0.25	0.74±0.35	0.77±0.32	2.04±0.23	ND

* Mean ± S.E. of mean for 5 root pieces, averaged along the lengths of root pieces.

** Not determined; in these roots the mycelium and the darkly pigmented structures of *M. bolleyi* did not permit an accurate assessment.

Table 3.14 Numbers of living cortical cell layers outside of the endodermis in sterile detached root pieces placed on colonies of *M. bolleyi*, or on PDA or on mineral nutrient agar after pre-incubation for 2 days on mineral nutrient agar and sampled after 1-5 days; assessments based on staining with neutral red-plasmolysis (NR-Pl) or acridine orange (AO).*

Days of incubation**	I N C U B A T I O N O N					
	Colonies of *M. bolleyi*.		P D A		Min. Agar.	
	A O	NR-Pl	A O	NR-Pl	A O	NR-Pl
1	N D	3.89±0.13	3.76±0.39	4.68±0.30	3.82±0.07	N D
2	1.07±0.39	2.10±0.20	1.64±0.42	2.43±0.64	2.70±0.29	N D
3	0.99±0.27	1.22±0.32	2.58±0.25	3.36±0.06	2.04±0.23	N D
4	0.61±0.21	0.73±0.19	2.58±0.61	2.79±0.75	2.17±0.13	1.80±0.22
5	0.00	0.00	N D	N D	1.23±0.27	1.01±0.08

* Mean ± S.E. of mean for 5 root pieces, averaged along the lengths of root pieces.

** Not including the 2-day pre-incubation.

N D Not done.

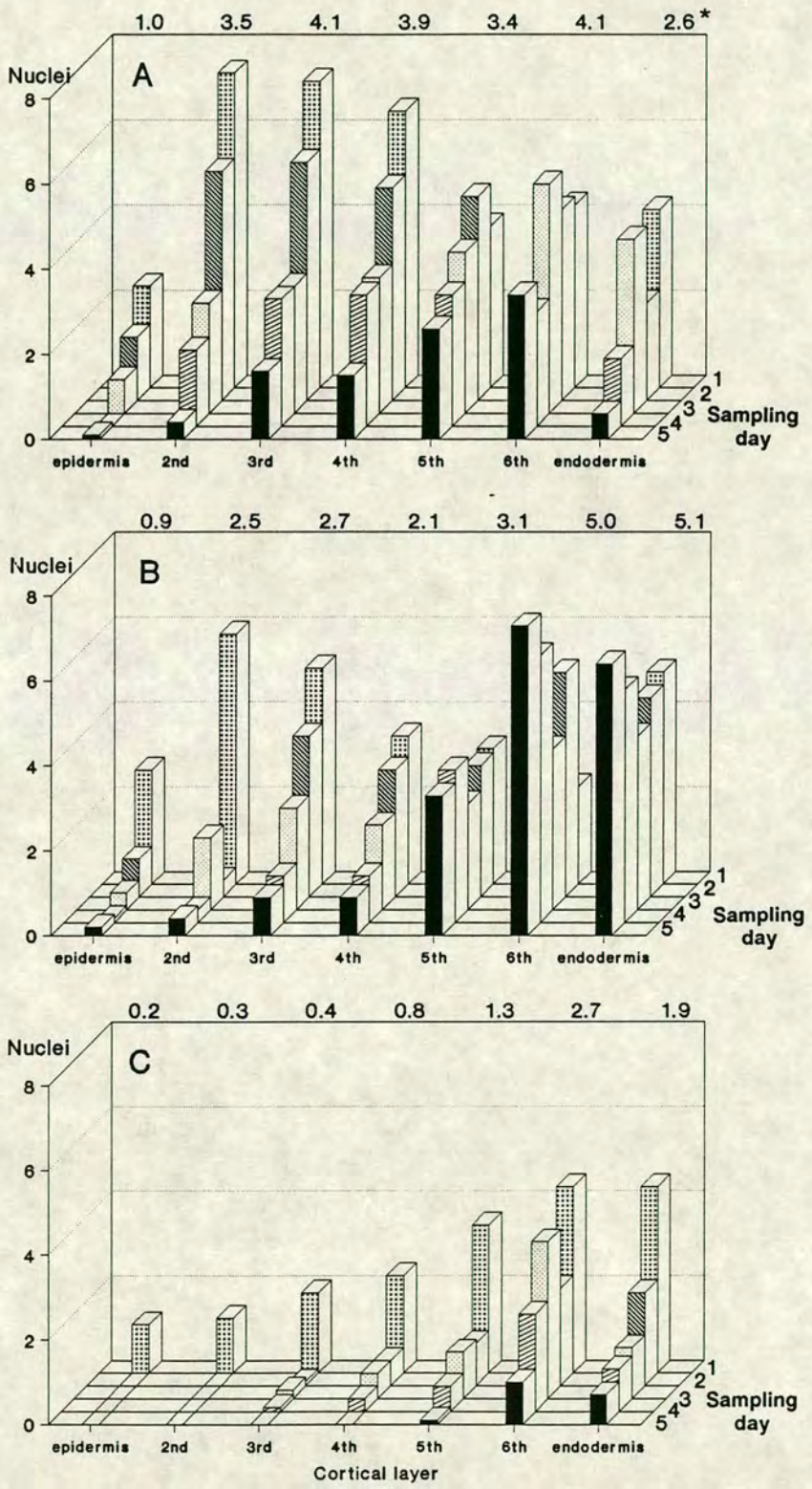
Table 3.15 Numbers of living cortical cell layers outside of the endodermis in sterile detached root pieces placed on colonies of *M. bolleyi*, or on PDA or mineral nutrient agar, after preincubation for 4 days on mineral nutrient agar and sampled after 1-5 days; assessments based on staining with neutral red-plasmolysis (NR-Pl) or acridine orange (AO).*

Days of incubation**	I N C U B A T I O N O N Colonies of *M. bolleyi*.		PDA		Min. Agar.	
	A O	NR-Pl	A O	NR-Pl	A O	NR-Pl
1	1.70±0.15	1.53±0.18	1.30±0.17	1.46±0.28	2.04±0.23	N D
2	0.37±0.19	0.56±0.23	1.51±0.21	1.85±0.21	2.17±0.13	1.80±0.22
3	0.17±0.11	0.31±0.15	0.86±0.12	1.48±0.13	1.23±0.27	1.01±0.08
4	N D	0.11±0.11	0.67±0.12	1.15±0.17	0.59±0.04	0.90±0.12
5	N D	0.00	0.78±0.14	1.15±0.05	0.71±0.12	0.88±0.14

* Mean ± S.E. of mean for 5 root pieces, averaged along the lengths of root pieces.

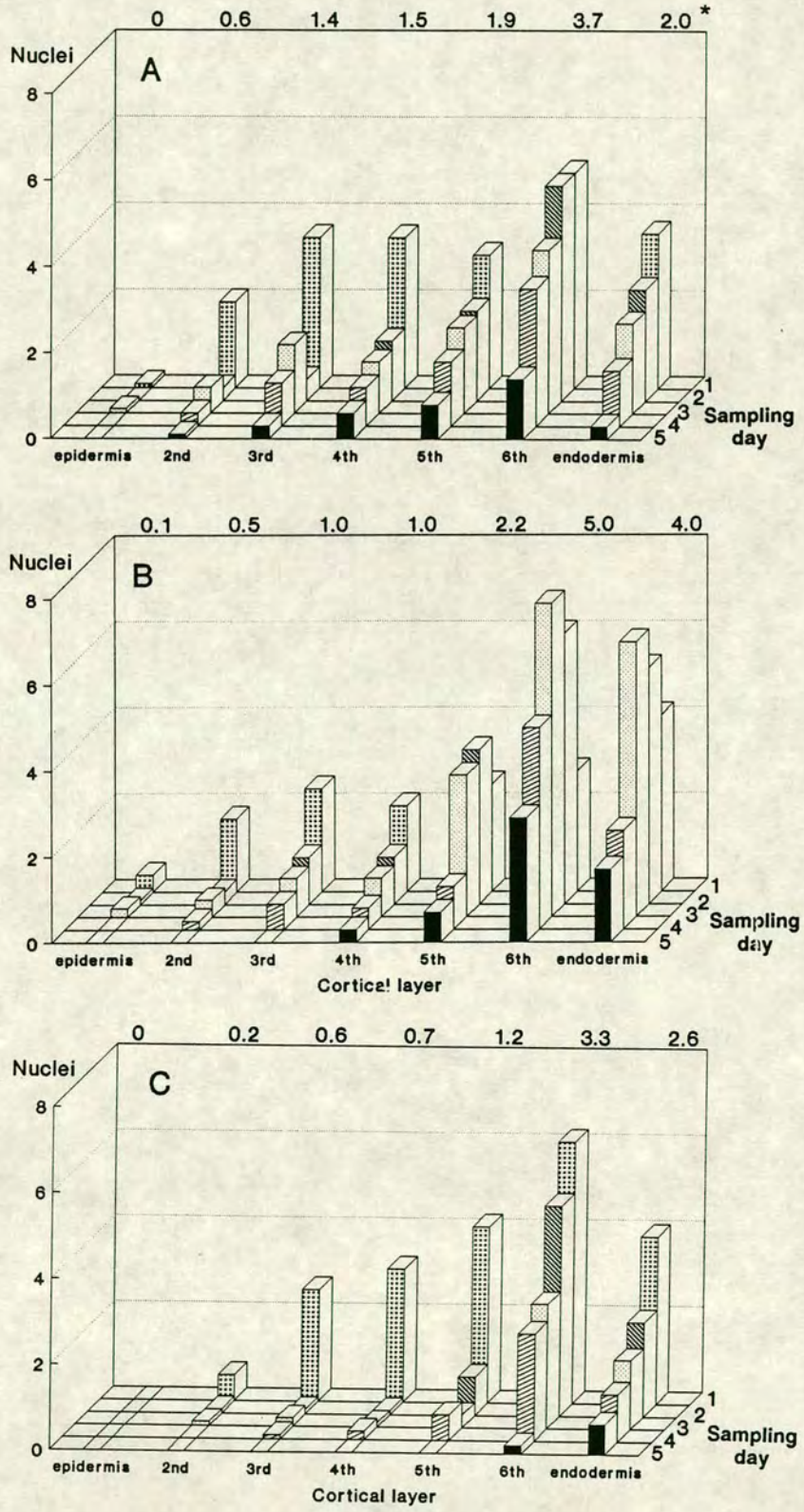
** Not including the 4-day pre-incubation.

Figure 3.9. Mean number of nuclei in each cortical cell layer of 50 μm sections from wheat root pieces freshly excised from seedlings and placed for 1 to 5 days on PDA (A), on mineral nutrient agar (B) or colonies of *M. bolleyi* (C).



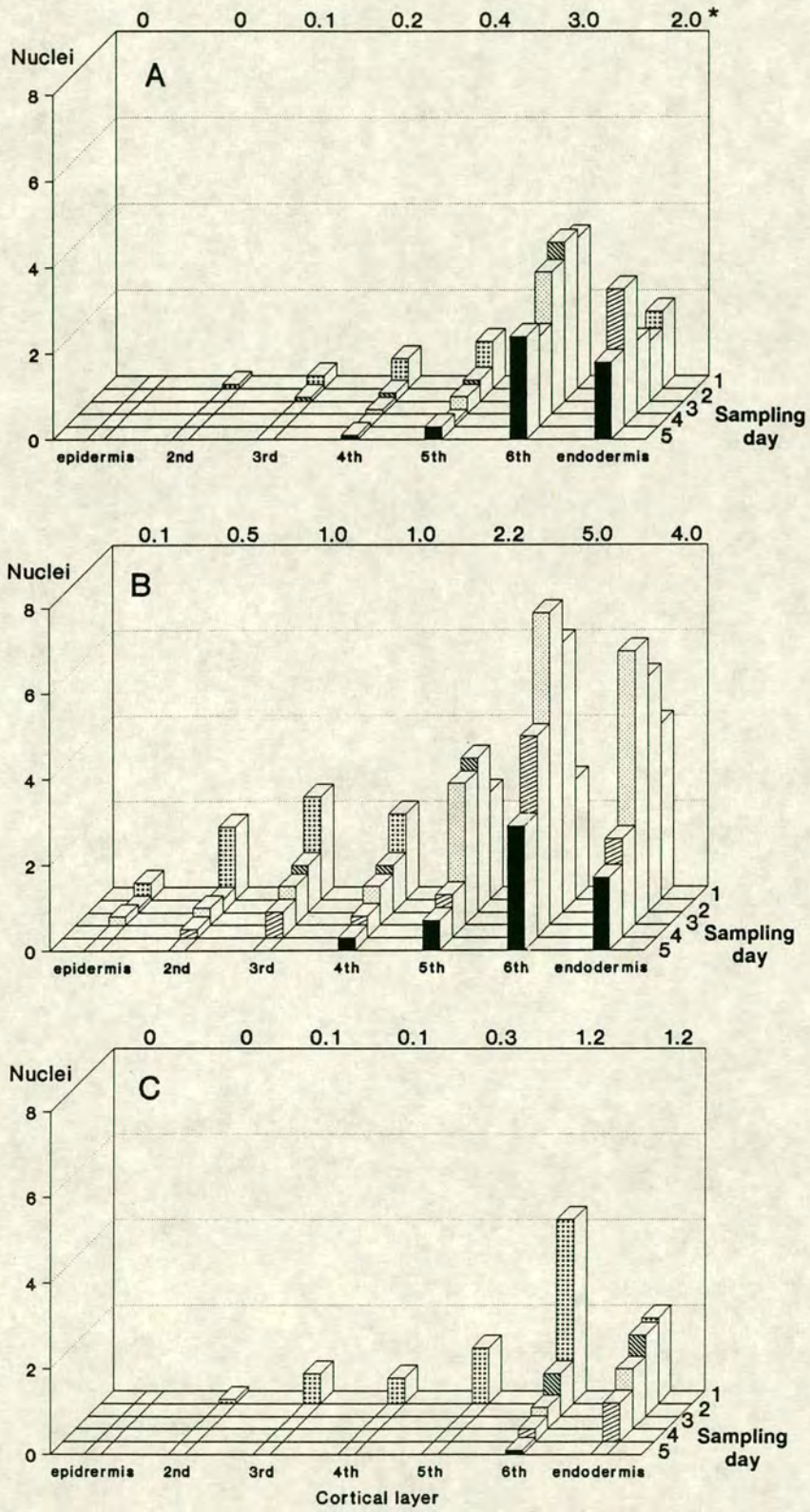
* Numbers above the histograms are means of all sampling days.

Figure 3.10. Mean number of nuclei in each cortical cell layer of 50 μm sections from wheat root pieces pre-incubated for 2 days on MA, then placed for 1 to 5 days on PDA (A), on mineral nutrient agar (B) or colonies of *M. bolleyi* (C).



* Numbers above the histograms are means of all sampling days.

Figure 3.11. Mean number of nuclei in each cortical cell layer of 50 µm sections from wheat root pieces pre-incubated for 4 days on MA, then placed for 1 to 5 days on PDA (A), on mineral nutrient agar (B) or colonies of *M. bolleyi* (C).



* Numbers above the histograms are means of all sampling days.

Figure 3.12. Relationship between assessments of living cortical cell layers (max.6) of wheat root pieces using neutral red staining followed by plasmolysis (NR-PI) and staining of nuclei with acridine orange (AO), in 50 μm sections of the same roots, (A) non-inoculated roots; (B) roots inoculated with *M. bolleyi.*

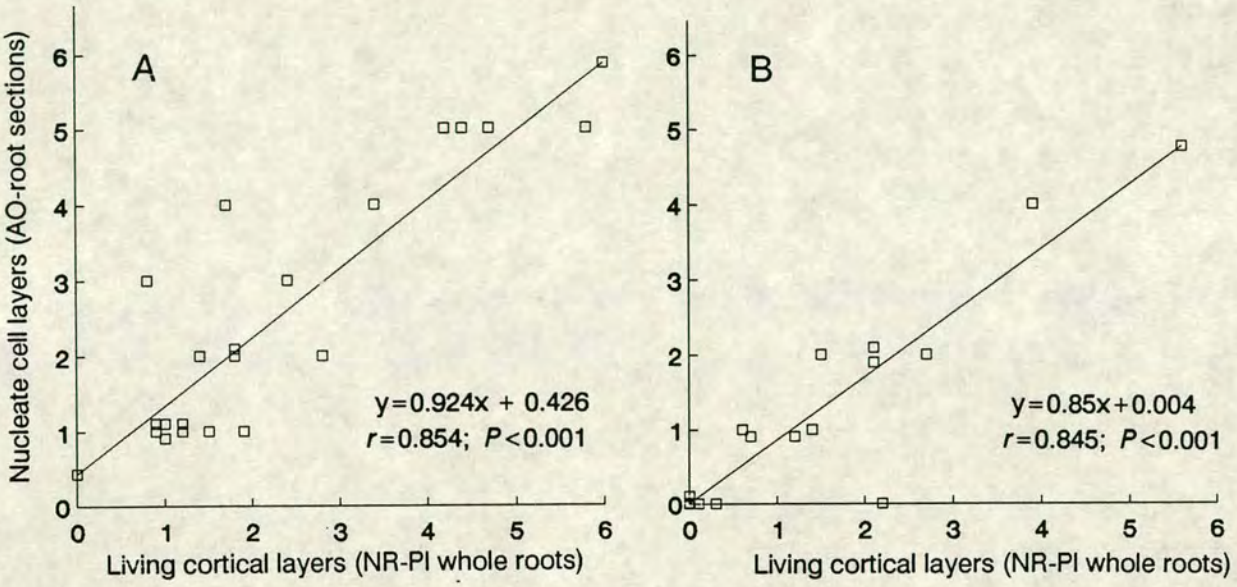


Figure 3.13. Relationship between number of cell layers of wheat root pieces penetrated intracellularly by hyphae of *M. bolleyi* and numbers of anucleate cell layers of control root pieces, non-inoculated but pre-incubated and incubated on MA for the same times (A), and of the same inoculated root pieces (B). Data pooled from all pre-incubation and sampling times.

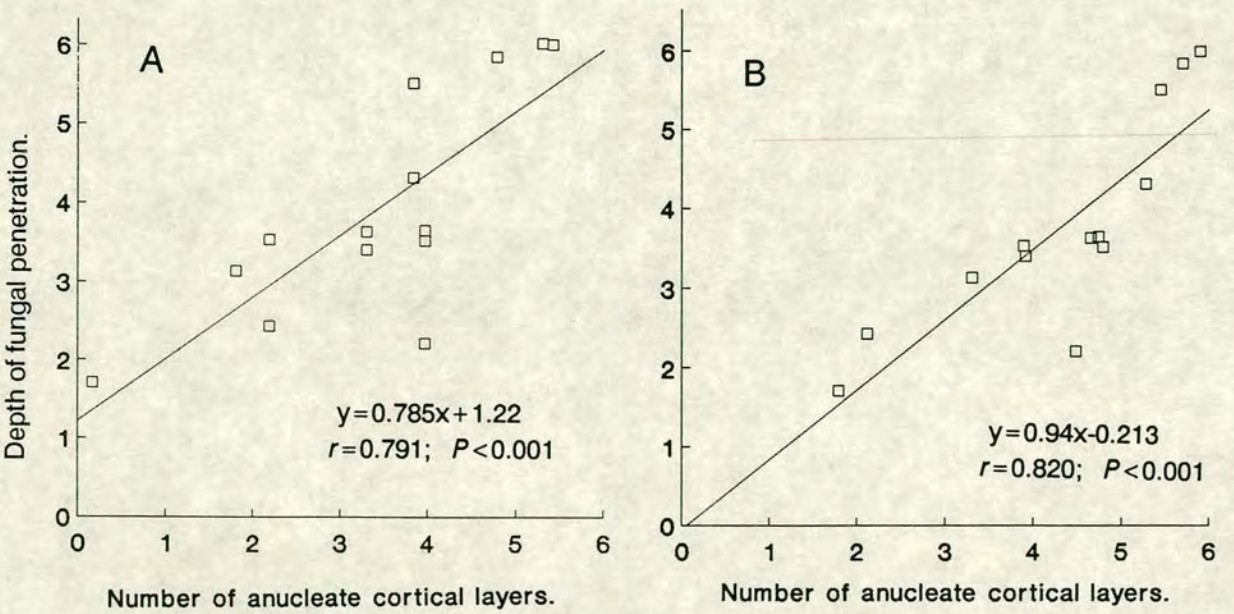
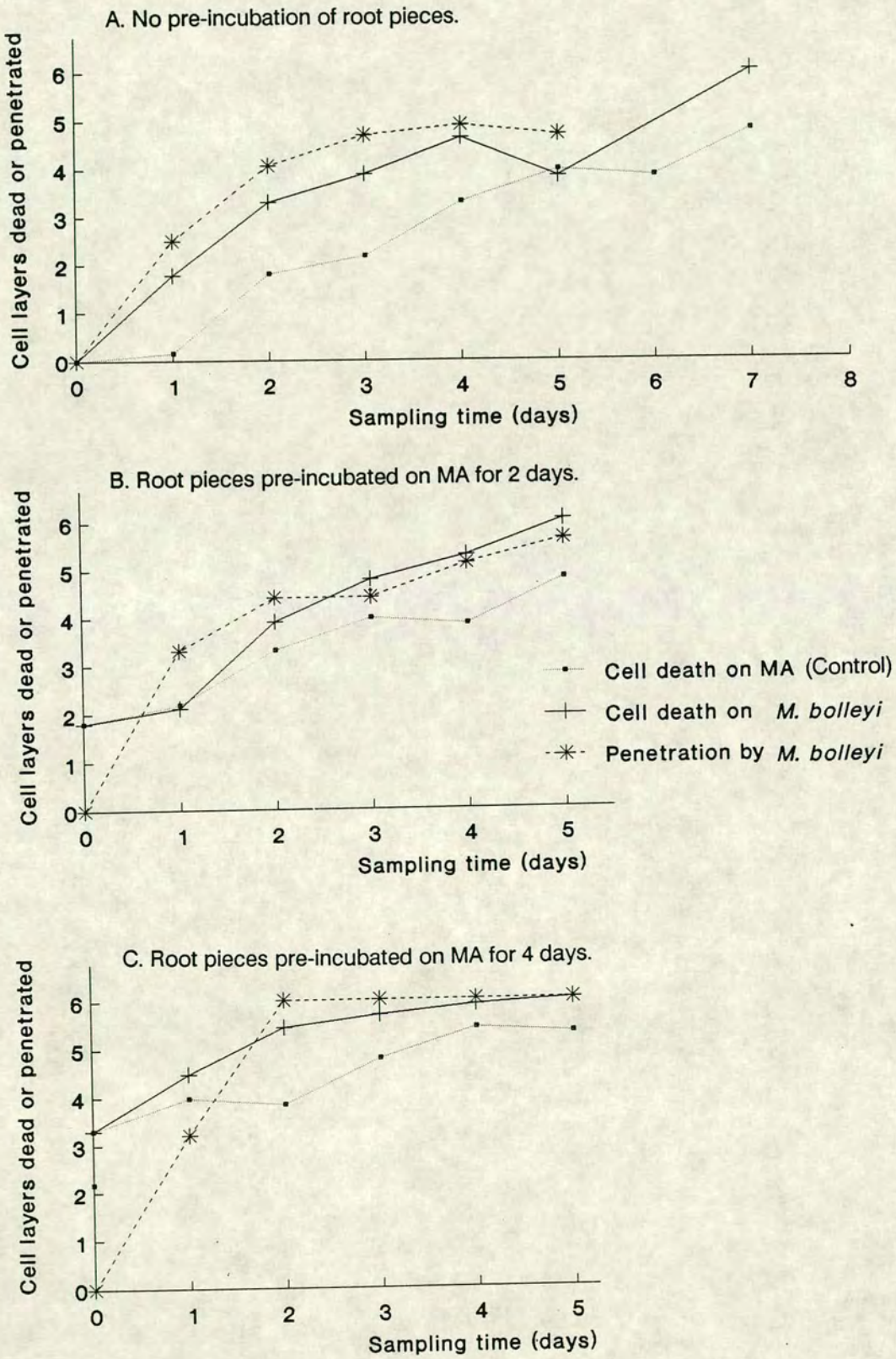
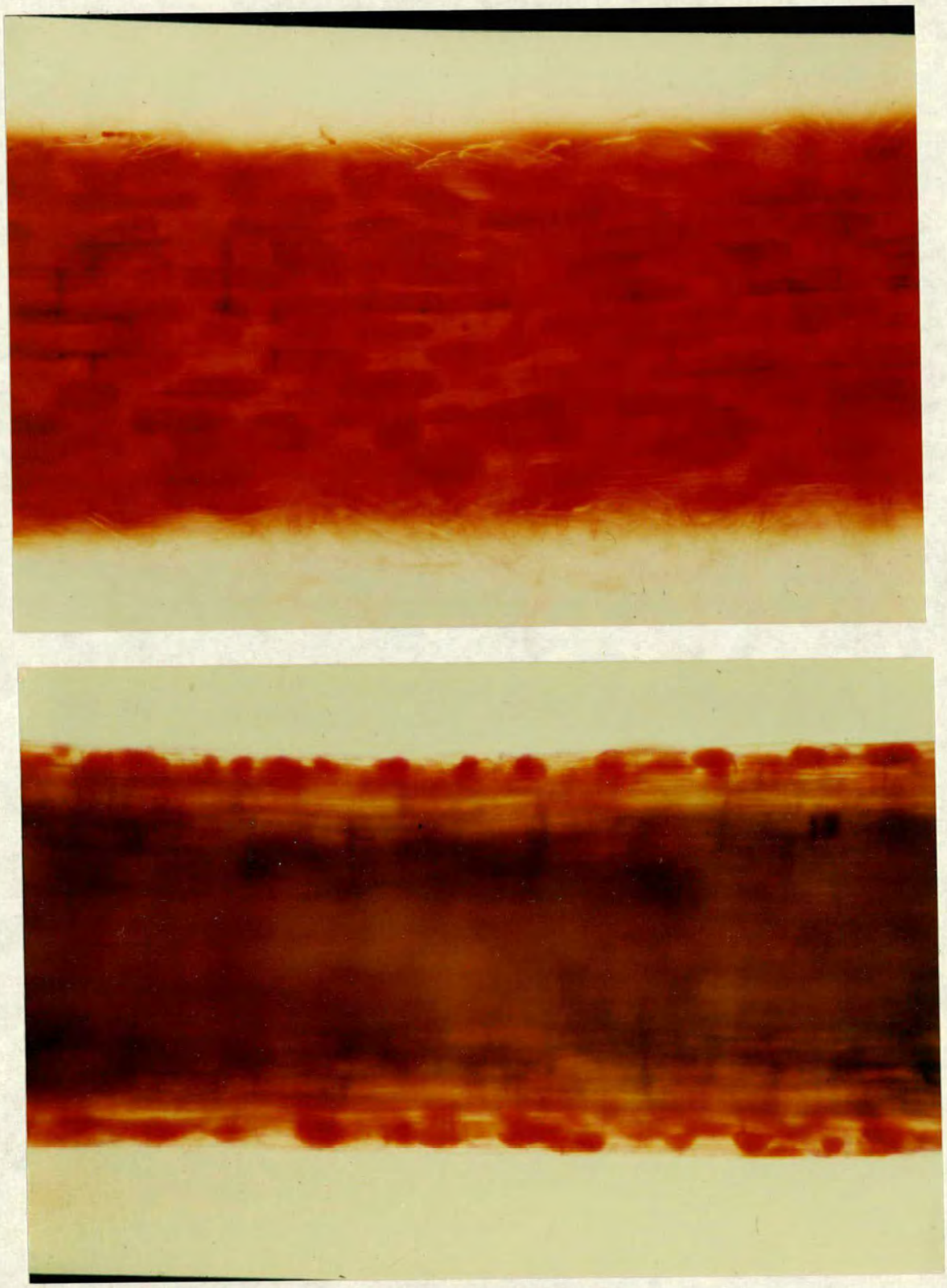


Figure 3.14. Relationship between numbers of anucleate cell layers in control (uninoculated) wheat root pieces and cell layers anucleate or penetrated intracellularly by hyphae when root pieces were placed on colonies of *M. bolleyi* and sampled at equivalent times.

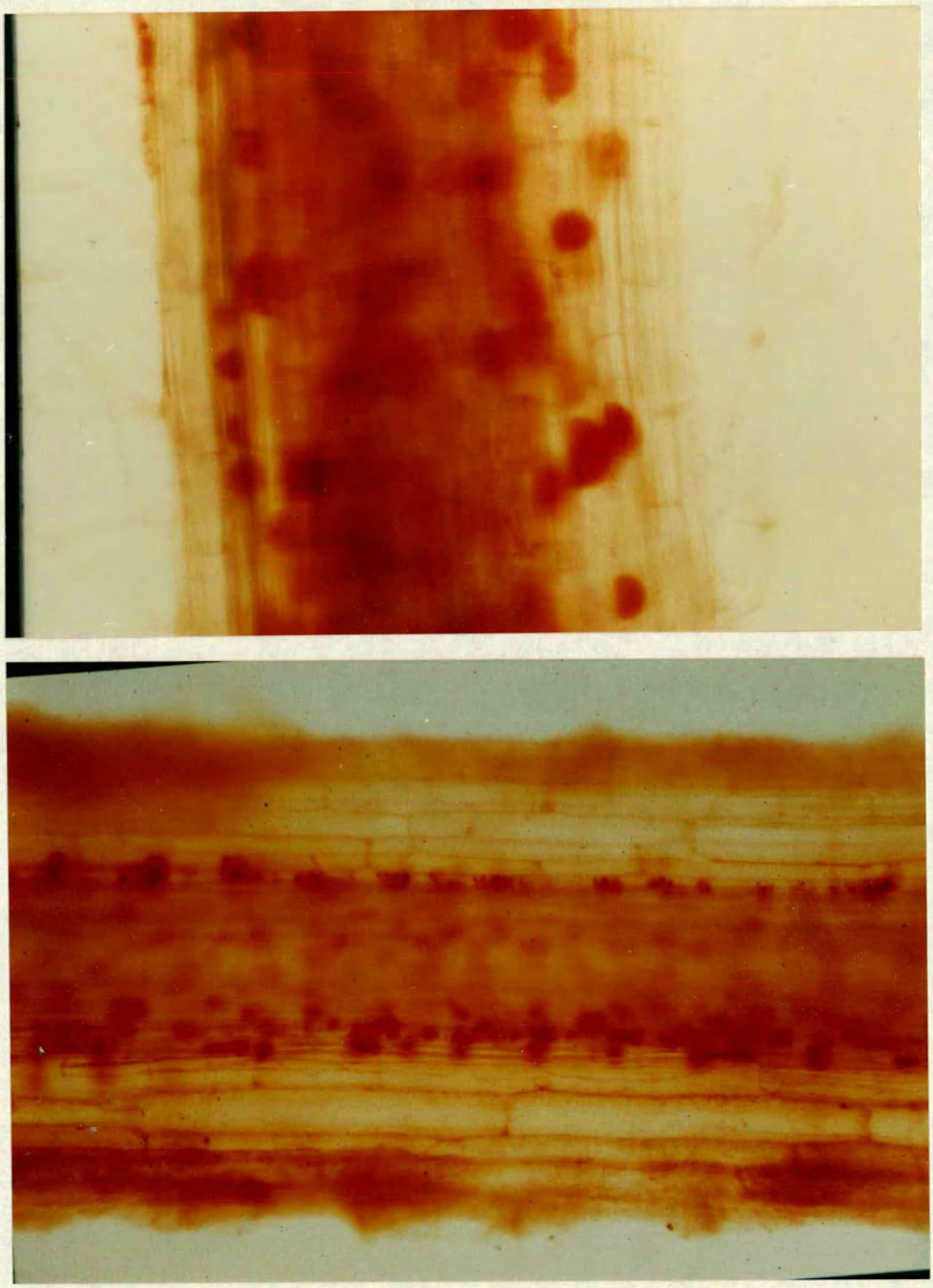




Figures 3.15. & 3.16. Neutral red staining of uninoculated wheat roots.

3.15. Root as viewed from the upper surface.

3.16. Equivalent root after part of the cortex was scraped away to reveal cortical cells in para-longitudinal section.



Figures 3.17, & 3.18. NR staining of wheat roots.

3.17. Root piece incubated for 2 days on mineral agar, showing loss of staining and plasmolytic ability of outer cortical cells.

3.18. Root piece incubated for 4 days on mineral agar then 3 days on a colony of *M. bolleyi*, showing restriction of plasmolytic ability to the inner cortex, and general staining of mycelial masses in the outer cortex.

of cortical cell death. To investigate this the data in Figures 3.9-3.11 were subjected to analysis of variance with three factors -"pre-incubation" (0, 2, or 4 days), "treatment" (*M. bolleyi*, PDA, or MA) and "sampling" (1-5 days after pre-incubation). The details of this analysis are shown in Appendix 1. The effect of pre-incubation was very highly significant (P=0.001) for all cortical layers except the sixth layer from the root surface (P=0.05). But there was no effect of pre-incubation on death of the endodermis in the experiment as a whole.

3.10.1.3. Effects of inoculation with *M. bolleyi*

M. bolleyi substantially increased the amount of cell death, especially in the outer cortical cell layers of the roots that were not pre-incubated on MA and in the inner cortex of roots that were pre-incubated for 2 and 4 days (Tables 3.13 - 3.15; Figures 3.9-3.11). Analysis across the pre-incubation and sampling times (Appendix 1), based on counts of nuclei in root sections, showed that the effect of *M. bolleyi* was very highly significant (P=0.001) for cell layers 2 to 7 counting the epidermis as "1", and highly significant for the outermost cell layer (P=0.01). There was no significant difference between the two controls (PDA and MA) except in respect of senescence of the fourth cell layer from the root surface (P=0.01), which cannot be explained.

3.10.1.4. Effect of sampling time

The root cortex showed an increasing degree of senescence with increasing time of incubation in all treatments, as was expected. The analysis of this effect is shown in Appendix 1.

3.10.1.5. Depth of invasion by *M. bolleyi*

As shown in Table 3.16 *M. bolleyi* invaded deeper into the root pieces at some points around their circumference than at other points, almost certainly depending on which part of the root piece was in contact with the fungal colony. The minimum and maximum were recorded, together with the average for six sectors that were assessed in each root section.

Considering the results for freshly excised root pieces (0 days pre-incubation) it is seen that the fungus invaded a mean of 1.7 cell layers after 1 day and just over 3 cell layers after 2 days but its rate of further penetration then slowed or halted, presumably owing to host resistance. Both the depth of penetration in the first day and the rate of subsequent penetration were increased in root pieces pre-incubated for 2 or 4 days on MA. There was evidence of a temporary delay in further penetration of root pieces pre-incubated for 2 days, but rapid and progressive penetration of root pieces pre-incubated for 4 days. All these results are consistent with a view that the root cortex presented some resistance to penetration beyond the first two cortical cell layers but that this resistance decreased markedly after 4 days' pre-incubation of the root pieces. The relationship between depth of penetration by *M. bolleyi* (all values censored to a maximum of 6) and assessment of the number of living cortical cell layers in the same roots (based on AO staining) is shown in Fig. 3.13. From these data, pooled for all pre-incubation and sampling times, it is clear that the fungus penetrated up to the cell layer that was recorded as nucleate in any root section. To some degree this was because the fungus caused the death of cells that it penetrated, but the results in Table 3.16 demonstrate also that it was because the living cells deeper in the

Table 3.16 Number of cell layers penetrated by hyphae of *Microdochium bolleyi* in wheat root pieces, pre-incubated for different times and then sampled after 1 to 5 days on colonies of *M. bolleyi*.*

Pre-incubation time (days)		Time (days) of incubation on culture of *M. bolleyi*				
		1	2	3	4	5
0	Maximum**	2.5 ± 0.31	4.1 ± 0.19	4.7 ± 0.32	4.9 ± 0.26	4.7 ± 0.27
	Minimum	1.1 ± 0.38	2.0 ± 0.26	2.4 ± 0.13	2.5 ± 0.31	2.6 ± 0.35
	Average	1.7 ± 0.34	3.1 ± 0.26	3.5 ± 0.22	3.6 ± 0.28	3.6 ± 0.26
2	Maximum	3.3 ± 0.15	4.4 ± 0.16	4.4 ± 0.21	5.1 ± 0.07	6.9 ± 0.43
	Minimum	1.5 ± 0.24	2.2 ± 0.20	2.4 ± 0.21	3.3 ± 0.35	5.7 ± 0.67
	Average	2.4 ± 0.13	3.4 ± 0.13	3.5 ± 0.20	4.3 ± 0.15	6.4 ± 0.52
4	Maximum	3.2 ± 0.37	6.8 ± 0.11	6.6 ± 0.21	7.8 ± 0.31	8.4 ± 0.27
	Minimum	1.2 ± 0.26	4.6 ± 0.21	5.7 ± 0.27	6.8 ± 0.73	7.7 ± 0.38
	Average	2.3 ± 0.27	5.7 ± 0.10	6.1 ± 0.22	7.2 ± 0.32	7.9 ± 0.34

* Maximum and minimum refer to different points around the circumference of a root piece; average is derived from separate assessments of six sectors around the circumference.

** Mean ± S.E. of mean for 15 sections from each sample.

cortex restricted the degree of invasion by the fungus. The effects are demonstrated further in Fig. 3.14 where the depth of penetration by *M. bolleyi* is shown in relation to assessments of cortical cell death in uninoculated root pieces (on MA) pre-incubated and sampled at equivalent times. It is evident that the depth of penetration by the fungus was slightly in advance of the degree of cell death found in uninoculated root pieces.

3.10.1.6. Observations on the infection behaviour of *M. bolleyi* and on host responses to invasion

Observations of sections stained with trypan blue revealed that in many instances the hyphae were attached to or closely associated with the root. Also, hyphae were commonly seen in cross-sections in the grooves formed by the junctions of epidermal cells and apparently in a mucilaginous material in these sites. Hyphae were seen to penetrate the root cortex intercellularly by separating the root epidermal cells in a manner reminiscent of the development of a Hartig net in ectomycorrhizas (Fig. 3.21).

Longitudinally running intercellular hyphae were seen deeper in the root cortex of some root pieces. In roots with several dead cell layers these "runner hyphae" were abundant and were seen deep in the cortex, up to the endodermis. In roots with less cell death the intercellular hyphae were observed usually one layer in advance of intracellular penetration. Invaded or challenged root cells sometimes had developed lignitubers or other papillae that did not give a positive reaction for lignin with phloroglucinol. Additionally, the roots showed a pattern of general lignification (or suberisation) of the cortical cell walls detectable with phloroglucinol (Table 3.17). Such lignification was weak in both

controls (PDA and MA) for all pre-incubation and sampling times; only a few cells had lignified walls and these cells were seen mainly in the second cortical layer (hypodermis). In contrast, roots placed on colonies of *M. bolleyi* after 0 or 2 days' pre-incubation showed extensive lignification for most cortical cell layers and roots placed on fungal colonies after 4 days' pre-incubation showed lignification mainly of the inner cortical cell layers. The reason for this was that the outer cell layers were dead or dying and evidently unable to react to the presence of the fungus.

Examination of sections from uninoculated root pieces by fluorescence microscopy showed that the vascular system and endodermal cell walls exhibited a characteristic bright blue autofluorescence while the cortical cell walls autofluoresced a greenish-blue colour. In root pieces that had been left to age (incubated on PDA or MA) the greenish-blue colour was replaced by yellowish fluorescence.

Autofluorescence of the cell walls of root pieces (Table 3.18) was more extensive and generalised than lignification detected with phloroglucinol-HCl. Autofluorescence was initially seen mainly in the outer cortex of control root pieces, but it progressed inwards with time of pre-incubation and time of sampling in these roots. It was consistently present in the epidermis and hypodermis of control root pieces. In the presence of *M. bolleyi* the intense wall fluorescence was seen much deeper in the cortex than in controls, particularly in the early sampling times and with the shorter pre-incubation times. From all these results it seems that the fluorescing substances are accumulated in the cell walls as the cells senesce irrespective of challenge by the fungus.

Table 3.17 Occurrence of lignification reactions (assessed with phloroglucinol-HCl) in walls of cells of wheat root pieces pre-incubated for different times on MA and then held for different times on plates of PDA, MA or colonies of *M. bolleyi* (Mb).

Cortical cell layer	Pre-incubation (days)	0										2										4									
	Treatment	MA					PDA					Mb					MA					PDA					Mb				
	Sampling time (days)	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
1		-	-	0	0	0	0	0	0	0	0	0	0	*	0	*	0	0	0	0	0	0	0	0	0	-	0	*	0	0	0
2		-	-	+	+	+	+	*	+	+	+	*	*	*	*	*	+	+	+	0	0	0	*	+	0	-	+	*	*	*	+	*	0	*	0	0	*	*	0	0	0	*	0	0	0	0
3		-	-	0	0	0	0	0	0	0	0	0	+	+	+	*	0	0	0	0	0	0	0	0	0	-	+	*	+	*	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4		-	-	0	0	0	0	0	0	0	0	0	+	+	+	*	0	0	0	0	0	0	+	0	+	-	+	*	+	+	+	0	0	0	0	0	+	0	0	0	+	0	0	0	0
5		-	-	0	0	0	0	0	0	0	0	0	+	+	+	*	0	0	0	0	0	0	+	0	+	-	+	*	+	+	+	0	0	0	0	0	+	0	0	0	+	*	*	*	0
6		-	-	0	0	0	0	0	0	0	0	0	+	+	+	*	0	0	0	0	0	0	0	0	0	-	+	*	+	+	+	0	0	0	0	0	0	+	0	+	0	*	*	*	*

0 No lignified cells.
\+ Some cells lignified.
* All cells lignified.
\- Not assessed.

Table 3.18 Occurrence of autofluoresence in walls of cells of wheat root pieces pre-incubated for different times on MA and then held for different times on plates of PDA, MA or colonies of *M. bolleyi* (Mb).

Cortical cell layer	Pre-incubation (days)	0										2										4									
	Treatment	MA					PDA					Mb					MA					PDA					Mb				
	Sampling time (days)	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
1		-	-	*	*	*	*	*	*	*	*	0	-	*	*	*	*	*	*	*	*	*	*	*	*	-	-	*	*	*	*	*	*	*	*	*	*	*	*
2		-	-	*	*	*	*	*	*	*	*	*	-	*	*	*	*	*	*	*	*	*	*	*	*	-	-	*	*	*	*	*	*	*	*	*	*	*	*
3		-	-	+	0	+	0	+	+	0	0	*	-	+	*	*	+	0	+	*	+	0	0	+	*	-	-	*	*	*	*	*	0	+	+	+	0	*	*	*	*
4		-	-	0	0	+	0	0	0	0	0	0	-	+	*	*	0	0	+	0	0	0	0	+	+	-	-	*	*	*	*	0	0	+	+	+	0	*	*	*	*
5		-	-	+	0	+	0	0	+	0	0	0	-	+	*	*	+	0	+	0	0	0	0	+	+	-	-	*	*	*	*	0	0	+	+	+	*	*	*	*	*
6		-	-	0	0	0	*	0	*	+	0	0	-	+	*	*	0	0	0	+	+	0	*	0	+	-	-	+	*	*	*	*	+	+	*	*	*	*	*	*	*

0 No yellow autofluorescence observed.
+ Some cell walls were autofluorescing.
* All cell walls were autofluorescing.
- Not done.

3.11. Invasion by *P. lycopersici* and *M. bolleyi*, of sterile roots of wheat and tomato attached to seedlings or detached from seedlings

This experiment was designed to study the effect of excision of roots on cortical senescence and implications for the abilities of two fungi to invade roots of "host" and non-host species. *M. bolleyi* is characteristically, although not exclusively, associated with graminaceous roots, whereas *Pyrenochaeta lycopersici* is characteristically associated with tomato.

Both attached or detached roots of wheat and tomato seedlings were used. The sterile seedlings were grown on plates of mineral nutrient agar (3-4 seedlings per plate) as described in Section 2.4. In those seedlings used to supply the excised roots, the first-formed root was cut at 5 and 25 mm from the tip and both the 5 mm apical part and the rest of the seedling were removed, leaving the 20 mm sub-apical lengths on the original agar plate. The seedlings which supplied the attached roots were left on the mineral nutrient agar and were inoculated, where appropriate, in the zone 5-25 mm from the tips of the first-formed roots.

Because the degree of invasion by parasitic or pathogenic fungi depends on the initial inoculum level, two types of inoculum were used, an agar block (high inoculum level) and a spore suspension (considered to provide a lower inoculum level). The agar-blocks (5x15 mm) were cut from the margins of colonies growing on PDA, 5 day old colonies being used for *M. bolleyi* and 8 day old colonies for *P. lycopersici*. The blocks were placed alongside and in contact with the root. Spores were taken from colonies on agar plates and a suspension was applied as a drop (0.05 ml containing 10^5 spores), which was spread across and in contact with the root with a sterile spatula.

Roots were sampled after 1, 2, 3, 4 and 5 days, with five replicates per treatment at each time. A 7-day sampling also was used for some treatments. The wheat roots were stained with AO, mounted on microscope slides and assessed for presence of nuclei in the cortical cells. Because the fungi sometimes obscured the nuclei the roots were also examined after sectioning with a freezing microtome. For this, the five replicate roots were pooled and a total 10 sections were taken at random for nuclear assessments as described in the previous experiment, and the nuclei present in each cortical layer were counted separately. Tomato root pieces were assessed for viability with NR-plasmolysis and then sectioned (50 μm sections pooled from the five replicates).

Sections from wheat and tomato roots were stained with trypan blue and assessed for the depth of fungal penetration (ten sections pooled for each sampling-treatment); others kept in 70% alcohol were stained with phloroglucinol and assessed for lignification or were examined unstained for autofluorescence, using a fluorescence microscope.

3.11.1. Results

3.11.1.1. Attached and detached wheat roots in the presence or absence of *M. bolleyi*

As shown in Table 3.19, cortical cell death occurred in uninoculated roots and, overall, it was faster in detached than in attached roots. It was enhanced by inoculation with *M. bolleyi*, but only in the detached roots, and the effect was greater for agar inocula than for spore inocula which had a significant effect only at the 5-day sampling. Because the data in this and in the following Tables showed a slight heteroscedasity they were transformed to

log(X+1) before analysis of variance. The results of assessments of nuclear numbers in transverse sections of the roots (Fig. 3.19) confirmed the findings from assessment of "whole" roots, i.e. when the numbers of nucleate cell layers were assessed by focusing through the cortex.

For "attached" roots *M. bolleyi* (agar inocula) had only a small effect on the rate of nuclear deletion from individual cortical cell layers, whereas for detached roots it had a greater effect, extending to the endodermis with increasing time of incubation, the effect being statistically significant for the hypodermis (P=0.001) when analysed across times of incubation (Appendix 2).

3.11.1.2. Attached and detached wheat roots in the presence or absence of *P. lycopersici*

As shown in Table 3.20, *P. lycopersici* significantly reduced the cortical viability of wheat roots when applied as agar blocks, and this effect was seen for both attached and detached roots. However, the spore inocula of *P. lycopersici* did not enhance the rate of cell death at any time; on the contrary, for reasons that are unclear, spore inocula significantly reduced the rate of cell death, the effect being especially marked for detached roots. Again, the analysis of numbers of nuclei in transverse sections confirmed these findings (Fig. 3.20). But, when analysed across sampling times, *P. lycopersici* (agar inocula) had a significant effect in enhancing the rate of root cortical death only in the hypodermis and third cortical cell layers of detached roots. Spore inocula significantly reduced the rate of RCD only in the third cell layer of detached roots (Appendix 3).

Table 3.19 Numbers of nucleate cortical cell layers in wheat roots attached to or detached from seedlings, in the presence or absence of *M. bolleyi* (Mb, agar block or spore inocula).*

SAMPLING DAY	CONTROL	Mb-AGAR	Mb-SPORES	ROW MEAN
	R O O T S	A T T A C H E D		
1	5.8 (0.830)	5.9 (0.836)	5.9 (0.838)	5.8 (*0.835*)
2	5.5 (0.816)	5.2 (0.795)	5.5 (0.814)	5.4 (*0.808*)
3	5.1 (0.788)	3.8 (0.682)	5.5 (0.712)	4.8 (*0.761*)
4	4.6 (0.746)	4.1 (0.708)	4.8 (0.767)	4.5 (*0.740*)
5	3.7 (0.667)	3.6 (0.659)	4.5 (0.736)	3.9 (*0.687*)
Column mean	4.9 (<u>0.769</u>)	4.5 (<u>0.736</u>)	5.2 (<u>0.793</u>)	4.9 (**0.766**)
	R O O T S	D E T A C H E D		
1	5.7 (0.827)	4.9 (0.768)	5.9 (0.838)	5.5 (*0.811*)
2	4.8 (0.764)	4.4 (0.729)	5.5 (0.813)	4.9 (*0.769*)
3	3.8 (0.683)	3.2 (0.617)	4.2 (0.714)	3.7 (*0.671*)
4	3.2 (0.621)	2.2 (0.499)	2.7 (0.562)	2.7 (*0.561*)
5	2.9 (0.591)	1.0 (0.288)	1.5 (0.385)	1.8 (*0.421*)
Column mean	4.1 (<u>0.697</u>)	3.1 (<u>0.580</u>)	4.0 (<u>0.662</u>)	3.7 (**0.647**)

* Means of five roots, assessed with AO, and log (X+1) in parentheses for statistical analysis as follows.

	S.E.D.	L.S.D.(1%)
Comparison between any two single means	0.030	0.080
Comparison between any two means of sampling days (italicized)	0.018	0.046
Comparison between any two means of treatments (underlined)	0.014	0.036
Comparison between the two grand means (bold)	0.008	0.020

Table 3.20 Numbers of living cortical cell layers in wheat roots attached to or detached from seedlings, in the presence or absence of *P. lycopersici* (Pl, agar block or spore inocula).*

SAMPLING DAY	CONTROL	Pl-AGAR	Pl-SPORES	ROW MEAN
	R O O T S		A T T A C H E D	
1	5.5 (0.815)	5.0 (0.778)	5.9 (0.837)	5.5 (*0.810*)
2	4.9 (0.774)	3.6 (0.655)	5.3 (0.800)	4.6 (*0.743*)
3	4.7 (0.754)	3.1 (0.607)	5.1 (0.783)	4.3 (*0.714*)
4	4.0 (0.698)	3.8 (0.667)	4.9 (0.772)	4.2 (*0.715*)
5	4.3 (0.722)	2.0 (0.464)	4.1 (0.704)	3.5 (*0.630*)
Column mean	4.7 (<u>0.752</u>)	3.5 (<u>0.653</u>)	5.0 (<u>0.779</u>)	4.4 (**0.722**)
	R O O T S	D E T A C H E D		
1	5.8 (0.835)	5.1 (0.785)	6.0 (0.845)	5.6 (*0.822*)
2	4.8 (0.760)	3.1 (0.607)	5.3 (0.798)	4.4 (*0.721*)
3	3.8 (0.675)	2.1 (0.482)	4.8 (0.765)	3.6 (*0.640*)
4	3.9 (0.691)	2.3 (0.512)	4.8 (0.762)	3.7 (*0.654*)
5	3.2 (0.622)	1.8 (0.426)	4.4 (0.727)	3.1 (*0.592*)
Column mean	4.3 (<u>0.716</u>)	2.9 (<u>0.562</u>)	5.0 (<u>0.779</u>)	4.1 (**0.686**)

* Means of five roots, assessed with AO, and log (X+1) in parentheses for statistical analysis as follows.

	S.E.D.	L.S.D.(1%)
Comparison between any two single means	0.039	0.101
Comparison between any two means of sampling days (italicized)	0.022	0.058
Comparison between any two means of treatments (underlined)	0.017	0.045
Comparison between the two grand means (bold)	0.010	0.026

Table 3.21 Numbers of living cortical cell layers in tomato roots attached to or detached from seedlings, in the presence or absence of *P. lycopersici* (Pl, agar block or spore inocula)*.

SAMPLING DAY	CONTROL	Pl-AGAR	Pl-SPORES
	R O O T S	A T T A C H E D	
1	4.3(0.720)	mainly dead**	4.9(0.770)
2	4.4(0.734)	0.0	4.8(0.760)
3	4.2(0.716)	N D	4.4(0.733)
4	4.5(0.742)	N D	3.7(0.668)
5	4.4(0.732)	N D	4.0(0.702)
7	4.2(0.719)	N D	1.1(0.232)
Mean	4.3 **(0.727)**		3.8 **(0.644)**
	R O O T S	D E T A C H E D	
1	4.7 (0.759)	0.4	4.7(0.757)
2	4.7 (0.754)	0.0	4.1(0.709)
3	4.2 (0.710)	N D	4.2(0.714)
4	4.1 (0.709)	N D	1.0(0.227)
5	4.4 (0.732)	N D	0.0(0.000)
7	4.3 (0.724)	N D	0.0(0.000)
Mean	4.4 **(0.729)**		2.4 **(0.401)**

* Means of five roots, assessed with NR-plasmolysis, and log (X+1) in parentheses for statistical analysis as below.

** Quantitative assessment not possible; most of the root areas not obscured by fungal structures had mainly dead cells.

	S.E.D.	L.S.D.(1%)
Comparison between any two single means	0.064	0.215
Comparison between any two means of treatments (bold)	0.026	0.087

Table 3.22 Numbers of living cortical cell layers in tomato roots attached to or detached from seedlings, in the presence or absence of *M. bolleyi* (Mb, agar block or spore inocula).*

SAMPLING DAY	CONTROL	Mb-AGAR	Mb-SPORES
	R O O T S	A T T A C H E D	
1	4.9 (0.769)	5.0	4.9 (0.755)
2	4.8 (0.766)	alive**	4.7 (0.773)
3	4.9 (0.769)	alive	4.2 (0.718)
4	4.4 (0.733)	alive	4.9 (0.771)
5	4.7 (0.757)	alive	4.8 (0.760)
7	4.4 (0.722)	alive	4.9 (0.771)
Mean	4.6 (**0.753**)		4.8 (**0.761**)
	R O O T S	D E T A C H E D	
1	5.0 (0.778)	4.0 (0.700)	4.8 (0.760)
2	4.8 (0.766)	4.4 (0.743)	4.8 (0.761)
3	5.0 (0.778)	4.0 (0.714)	4.4 (0.733)
4	4.9 (0.770)	3.8 (0.675)	4.2 (0.713)
5	4.6 (0.751)	0.0 (0.000)	1.9 (0.373)
7	4.5 (0.744)	0.0 (0.000)	0.0 (0.000)
Mean	4.8 (**0.765**)	2.7 (**0.472**)	3.3 (**0.557**)

* Means of five roots, assessed with NR-plasmolysis, and log (X+1) in parentheses for statistical analysis as below.

** Quantitative assessment not possible; most of the root areas not obscured by fungal structures had mainly alive cells.

	S.E.D.	L.S.D. (1%)
Comparison between any two single means	0.046	0.154
Comparison between any two means of treatments (bold)	0.019	0.064

3.11.1.3. Attached and detached tomato roots in the presence or absence of *P. lycopersici*

As shown in Table 3.21, tomato roots assessed by NR-plasmolysis showed little cortical cell death over 7 days in the absence of inoculation; only some epidermal cells had died and there was no significant difference between attached and detached roots.

Inoculation with *P.lycopersici* (agar-block) had a drastic effect on the viability of cells in both attached or detached roots. Even after one day the fungus had killed almost all cortical cells. The spore inoculum of the same fungus had a delayed effect on root viability; by the fourth day it had killed most of the cortical cells in detached roots, and by the seventh day most of those in attached roots. Spores of this fungus germinated within one day , so the delayed effect of spores compared with the mycelial inocula could not be explained by germination time alone.

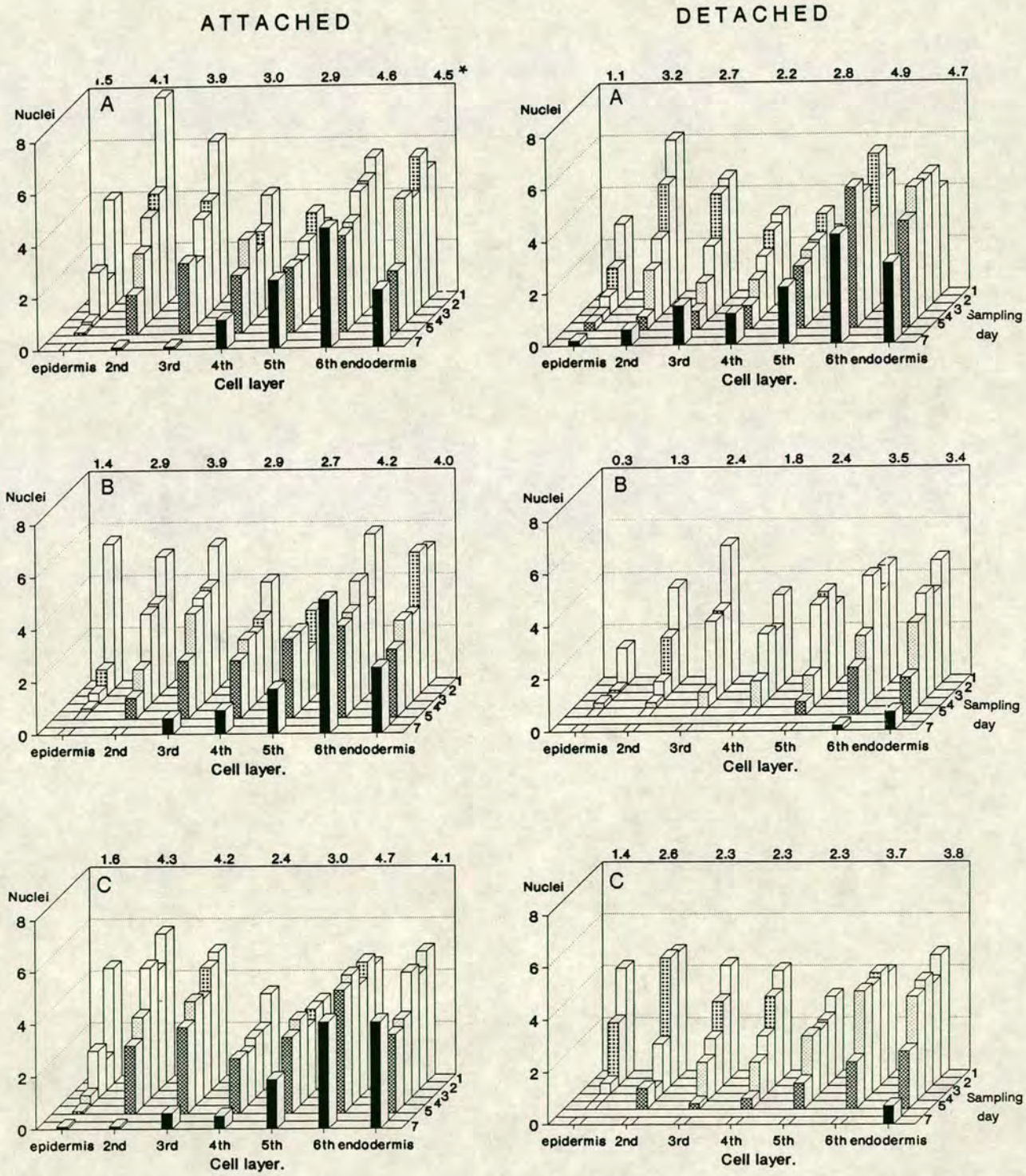
3.11.1.4. Attached and detached tomato root in the presence or absence of *M. bolleyi*

M. bolleyi had a less marked effect than did *P. lycopersici* on death of tomato root cortices (Table 3.22). In fact, there was no effect on attached roots but a significant effect of both spore and agar inocula on detached roots after 4 days. Assessments of attached roots in the presence of agar inocula were hindered by growth of *M. bolleyi* over the root surface, and many roots bore black sclerotium-like bodies of *M. bolleyi*. Nevertheless, all such roots had regions where the cortex could be seen to contain living cells.

3.11.1.5. Penetration of the wheat root cortex by the two fungi

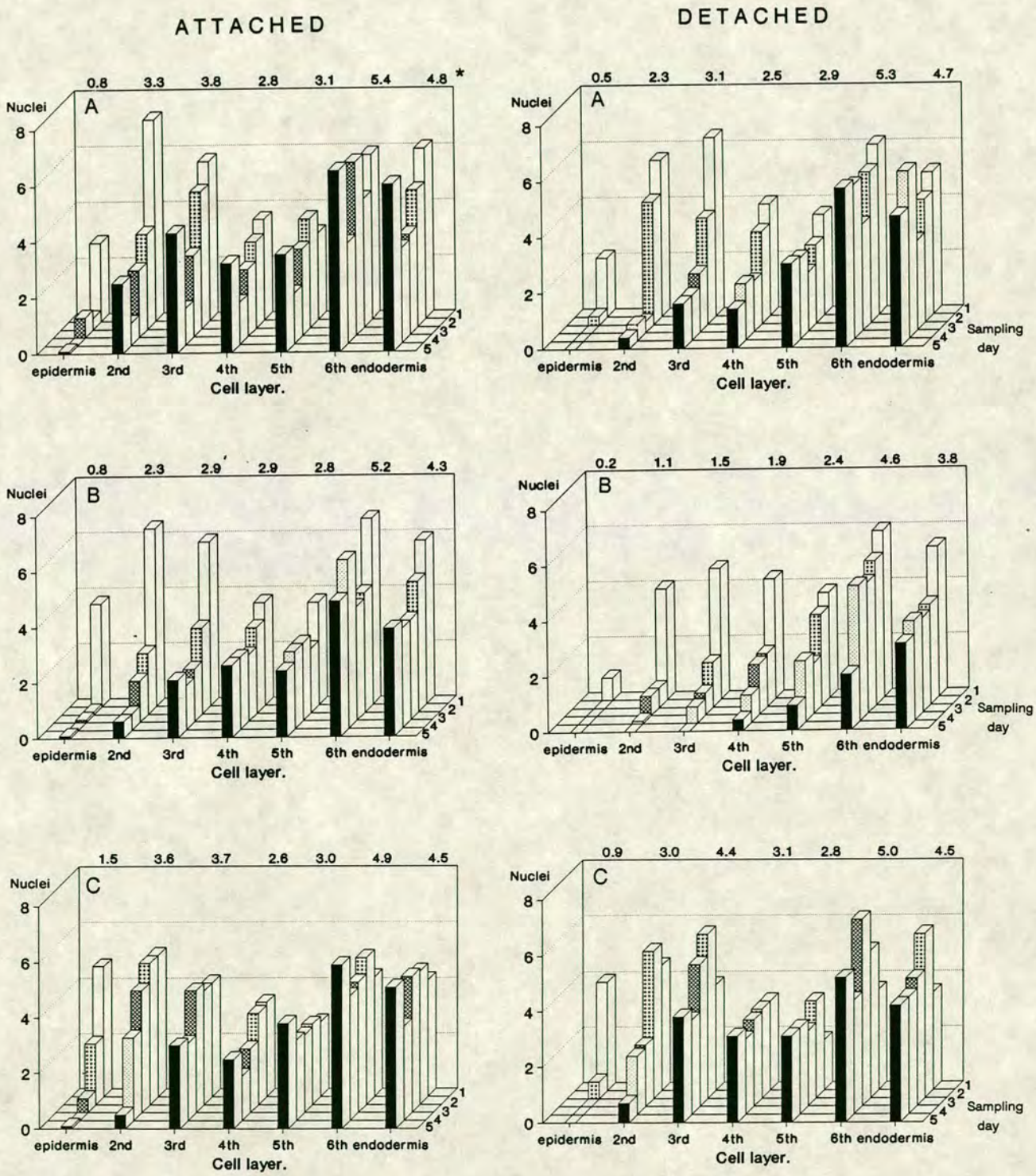
Tables 3.23 and 3.24 show the numbers of cell layers of wheat roots penetrated by *M. bolleyi* and *P. lycopersici*. For each root

Figure 3.19. Mean numbers of nuclei in each cortical cell layer and endodermis in transverse sections of wheat roots, attached or detached from seedlings, sampled after 1-5 days' incubation on MA. Non-inoculated (A), inoculated with *M. bolleyi* PDA-inocula (B) or spore inocula (C).



* Numbers above the histograms are means of all sampling days.

Figure 3.20. Mean numbers of nuclei in each cortical cell layer and endodermis in transverse sections of wheat roots, attached or detached from seedlings, sampled after 1-5 days' incubation on MA. Non-inoculated (A), inoculated with *P. lycopersici* PDA-inocula (B), or spore inocula (C).



* Numbers above the histograms are means of all sampling days.

section (10 per sample) a distinction was made between the deepest and the shallowest extent of penetration around the circumference of the section. Both fungi penetrated rapidly from agar inocula in the first one or two days, with little or no significant increase in penetration over the following days. Indeed *M. bolleyi* never penetrated beyond the third cell layer, on average, despite the fact that more than this number of cell layers had died when assessed by AO staining (Table 3.19). Except for the final (7th day), sampling time, there was no significant difference in degree of penetration of attached compared with detached roots by *M. bolleyi* from agar inocula. There was, however, a significantly and constantly greater degree of penetration of detached compared with attached roots by *M. bolleyi* from spore inocula. Of interest, *M. bolleyi* was seen to be present deeper in the cortex as intercellular hyphae than as intracellular hyphae, at all sampling times and with both types of inocula when the presence of intercellular hyphae enabled such comparisons to be made (Table 3.23 and Figures 3.23, 3.31). The depth of intercellular hyphae was recorded as, for example, 3 cell layers if they were present between the third and the forth cell layers. The intercellular hyphae were most common in detached roots, perhaps because the fungus had penetrated through their cut ends; nevertheless, they were also present in some attached roots. Invasion by *P. lycopersici* was more pronounced than that by *M. bolleyi* (Table 3.24). Again it reached the maximum depth by the second day from agar inocula and, unlike the situation for *M. bolleyi*, it was consistently deeper in detached roots than in attached wheat roots (compare Figures 3.25 and 3.26). The degree of penetration from spores of *P. lycopersici* was always less than from agar inocula.

3.11.1.6. Penetration of the tomato root cortex by the two fungi

Tomato roots were penetrated poorly by *M. bolleyi* with no significant differences between attached and detached roots at any single sampling time (Table 3.25). There was virtually no invasion from spore inocula of *M. bolleyi*. In contrast, *P. lycopersici* penetrated both attached and detached roots rapidly from agar inocula (Table 3.26 and Figures 3.27, 3.28). Invasion was poor or non-existent from spore inocula, except after 5 days when a low degree of invasion of intact roots was seen and there was complete invasion of detached roots.

3.11.1.7 Host responses to invasion

Lignification reactions (assessed with phloroglucinol-HCl) were not seen in the cortex of uninoculated roots, but occurred in the epidermis, hypodermis and third cortical cell layers of wheat roots inoculated with agar blocks of *M. bolleyi* (Table 3.27). The extent of these reactions was similar for attached and detached roots but the intensity in the hypodermis was greater (Fig. 3.32) in attached than detached roots. Virtually no such reaction was seen in roots inoculated with spores of *M. bolleyi*. Yellow autofluorescence of the walls of cortical cells was seen in control and inoculated roots, with relatively little difference in extent of the phenomenon between treatments but a greater intensity in inoculated roots than in uninoculated roots (Table 3.28).

Agar inocula of *P. lycopersici* induced a large degree of lignification (assessed by phloroglucinol-HCl) in attached roots but not in detached roots (Table 3.27), but spore inocula did not induce these reactions. Of particular interest is the induction by *P. lycopersici* in the attached wheat roots of large lignitubers

Table 3.23 Numbers of cell layers penetrated by hyphae in wheat roots attached to or detached from seedlings and inoculated with *Microdochium bolleyi* (agar block or spore inocula)*.

SAMPLING DAY		ATTACHED		DETACHED	
		AGAR BLOCK	SPORES	AGAR BLOCK	SPORES
1	Max.**	1.6±0.22	0.0±0.00	2.2±0.13	0.4±0.16
	Min.	0.1±0.10	0.0±0.00	0.8±0.20	0.0±0.00
	Average	0.7±0.10	0.0±0.00	1.6±0.11	0.1±0.04
2	Max.	2.1±0.10	0.9±0.23	2.2±0.13	0.7±0.15
	Min.	0.5±0.17	0.0±0.00	1.2±0.13	0.1±0.10
	Average	1.4±0.10 (1.6±0.18)	0.3±0.09	1.7±0.23 (2.2±0.28)	0.3±0.11
3	Max.	2.1±0.23	0.8±0.13	2.3±0.15	1.4±0.16
	Min.	0.8±0.25	0.1±0.10	1.3±0.15	0.8±0.13
	Average	1.5±0.21	0.3±0.09	1.9±0.11 (2.2±0.24)	1.2±0.13 (1.3±0.14)
4	Max.	2.5±0.17	0.1±0.15	2.7±0.15	1.5±0.31
	Min.	0.9±0.10	0.1±0.17	1.9±0.10	0.8±0.13
	Average	1.4±0.18	0.9±0.10	2.3±0.10 (3.4±0.34)	1.2±0.17 (1.8±0.37)
5	Max.	2.5±0.17	0.1±0.00	2.3±0.26	2.2±0.29
	Min.	0.8±0.13	0.0±0.00	1.6±0.16	1.5±0.17
	Average	1.6±0.08 (1.8±0.11)	0.6±0.05	2.1±0.09 (4.2±0.36)	1.8±0.19 (2.7±0.38)
7	Max.	2.0±0.15	1.1±0.18	3.1±0.14	2.1±0.10
	Min.	0.9±0.18	0.0 0.00	1.6±0.20	0.8±0.20
	Average	1.4±0.16 (2.1±0.13)	0.4±0.09 (0.6±0.22)	2.2±0.24 (4.6±0.21)	1.4±0.15 (4.0±0.41)

* Mean number of cell layers penetrated intracellularly ± S.E. of mean for 10 sections from each sample; in parentheses, mean depth (as cell layers) of intercellular hyphae (when present).

** Max. and min. = maximum and minimum depth of invasion around the circumference of each section; average = mean depth of penetration for 6 sectors around the circumference of the section.

Table 3.24 Numbers of cell layers penetrated by hyphae, in wheat roots attached to or detached from seedlings and inoculated with *Pyrenochaeta lycopersici* (agar block or spore inocula).*

SAMPLING DAY		ATTACHED		DETACHED	
		AGAR BLOCK	SPORES	AGAR BLOCK	SPORES
1	Max.**	2.0±0.33	0.0±0.00	4.2±0.63	0.0±0.00
	Min.	0.0±0.00	0.0±0.00	0.1±0.10	0.0±0.00
	Average	0.9±0.14	0.0±0.00	1.8±0.38	0.0±0.00
2	Max.	4.2±0.40	0.3±0.15	5.7±0.56	0.9±0.10
	Min.	1.1±0.16	0.0±0.00	1.9±0.38	0.0±0.00
	Average	2.4±0.27	0.5±0.03	3.7±0.48	0.4±0.06
3	Max.	3.1±0.23	1.2±0.13	5.4±0.40	1.7±0.30
	Min.	1.3±0.15	0.1±0.10	1.5±0.17	0.6±0.22
	Average	2.5±0.15	0.5±0.10	3.5±0.31	1.2±0.21
4	Max.	3.5±0.43	1.2±0.13	4.5±0.27	1.3±0.15
	Min.	1.4±0.16	0.0±0.00	1.8±0.33	0.3±0.15
	Average	2.4±0.25 (2.7±0.23)	0.7±0.08	3.1±0.17 (3.4±0.21)	0.8±0.09
5	Max.	2.7±0.41	0.9±0.28	3.7±0.52	1.2±0.20
	Min.	1.1±0.11	0.0±0.00	1.8±0.39	0.2±0.13
	Average	1.8±0.24 (2.1±0.13)	0.5±0.14	2.6±0.46 (2.7±0.46)	0.7±0.13
7	Max.	4.3±0.47			2.1±0.23
	Min.	1.3±0.17	N D	N D	1.1±0.18
	Average	2.6±0.21 (3.7±0.28)			1.6±0.15

* Mean number of cell layers penetrated intracellularly ± S.E. of mean for 10 sections from each sample; in parentheses, mean depth (as cell layers) of intercellular hyphae (when present).

** Max. and min. = maximum and minimum depth of invasion around the circumference of each section; average = mean depth of penetration for 6 sectors around the circumference of the section.

Table 3.25 Numbers of cell layers penetrated by hyphae in tomato roots attached to or detached from seedlings and inoculated with *M. bolleyi* (agar block or spore inoculum).*

SAMPLING DAY		ATTACHED		DETACHED	
		AGAR BLOCK	SPORES	AGAR BLOCK	SPORES
1	Max.**	0.9±0.10	0.0±0.00	1.1±0.10	0.0±0.00
	Min.	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00
	Average	0.3±0.06	0.0±0.00	0.5±0.04	0.0±0.00
2	Max.	0.8±0.13	0.0±0.00	0.9±0.10	0.2±0.13
	Min.	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00
	Average	0.4±0.04	0.0±0.00	0.4±0.07	0.1±0.07
3	Max.	0.8±0.13	0.0±0.00	1.0±0.00	0.0±0.00
	Min.	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00
	Average	0.2±0.06	0.0±0.00	0.4±0.07	0.0±0.00
4	Max.	1.0±0.00	0.3±0.15	1.1±0.10	0.6±0.16
	Min.	0.0±0.00	0.0±0.00	0.1±0.10	0.0±0.00
	Average	0.5±0.06	0.1±0.06	0.5±0.09	0.2±0.12
5	Max.	0.8±0.13	0.0±0.00	1.8±0.81	
	Min.	0.0±0.00	0.0±0.00	0.9±0.90	N D
	Average	0.3±0.07	0.0±0.00	1.2±0.87	
7	Max.	1.2±0.13		1.3±0.21	
	Min.	0.2±0.13	N D	0.1±0.10	N D
	Average	0.7±0.08		0.6±0.15	

* Mean number of cell layers penetrated intracellularly ± S.E. of mean for 10 sections from each sample; in parentheses, mean depth (as cell layers) of intercellular hyphae (when present).

** Max. and min. = maximum and minimum depth of invasion around the circumference of each section; average = mean depth of penetration for 6 sectors around the circumference of the section.

Table 3.26 Numbers of cell layers penetrated by hyphae, in tomato roots attached to or detached from seedlings and inoculated with *P. lycopersici* (agar block or spore inoculum).*

SAMPLING DAY		ATTACHED		DETACHED	
		AGAR BLOCK	SPORES	AGAR BLOCK	SPORES
1	Max.**	6.1±1.07	0.0±0.00	8.0±0.00	0.0±0.00
	Min.	5.1±1.41	0.0±0.00	8.0±0.00	0.0±0.00
	Average	5.7±1.20	0.0±0.00	8.0±0.00	0.0±0.00
2	Max.	7.1±0.59	0.0±0.00	8.0±0.00	0.0±0.00
	Min.	6.2±1.18	0.0±0.00	8.0±0.00	0.0±0.00
	Average	6.5±0.97	0.0±0.00	8.0±0.00	0.0±0.00
3	Max.		0.0±0.00		0.3±0.30
	Min.	N D	0.0±0.00	N D	0.0±0.00
	Average		0.0±0.00		0.1±0.13
4	Max.		0.3±0.15		0.3±0.15
	Min.	N D	0.0±0.00	N D	0.0±0.00
	Average		0.1±0.04		0.1±0.06
5	Max.		1.2±0.33		8.0±0.00
	Min.	N D	0.3±0.20	N D	8.0±0.00
	Average		0.5±0.23		8.0±0.00

* Mean number of cell layers penetrated intracellularly ± S.E. of mean for 10 sections from each sample; in parentheses, mean depth (as cell layers) of intercellular hyphae (when present).

** Max. and min. = maximum and minimum depth of invasion around the circumference of each section; average = mean depth of penetration for 6 sectors around the circumference of the section.

Table 3.27. Occurrence of lignification reactions (assessed with phloroglucinol-HCl) in walls of cells of wheat roots excised or attached to seedlings and uninoculated (control) or inoculated with *M. bolleyi* or *P. lycopersici* (agar blocks or spores), then sampled at different times.

Cell layer	Roots attached			Roots detached		
	Control Day	Agar Day	Spores Day	Control Day	Agar Day	Spores Day
	1 3 5	1 3 5 7	1 3 5 7	1 3 5	1 3 5 7	1 3 5 7
			M. bolleyi			
1	0 0 0	+ + + +	0 0 0 0	0 0 0	* + + +	0 0 0 0
2	0 0 0	* * * *	0 0 0 0	0 0 0	* + * +	0 + 0 0
3	0 0 0	0 + + +	0 0 0 0	0 0 0	+ 0 + +	0 0 0 0
4	0 0 0	0 + 0 0	0 0 0 0	0 0 0	0 0 0 0	0 0 0 0
5	0 0 0	0 0 0 0	0 0 0 0	0 0 0	0 0 0 0	0 0 0 0
6	0 0 0	0 0 0 0	0 0 0 0	0 0 0	0 0 0 0	0 0 0 0
7	* * *	* * * *	* * 0 *	* * *	* * * *	* * * *
			P. lycopersici			
1	0 0 0	0 0 0 0	0 0 0 -	+ 0 0	0 0 0 0	0 0 0 -
2	+ 0 0	+ + + *	0 0 0 -	0 0 0	0 0 0 0	0 0 0 -
3	0 0 0	0 + + +	0 0 0 -	0 0 0	0 0 0 0	0 0 0 -
4	0 0 0	0 + + +	0 0 0 -	0 0 0	0 0 0 0	0 0 0 -
5	0 0 0	0 + + +	0 0 0 -	0 0 0	0 0 0 0	0 0 0 -
6	0 0 0	0 + + +	0 0 0 -	0 0 0	0 0 0 0	0 0 0 -
7	* * *	* + + +	* + * -	* * +	* + + *	* * + -

0 No lignified cell walls.

\+ Some cell walls lignified.

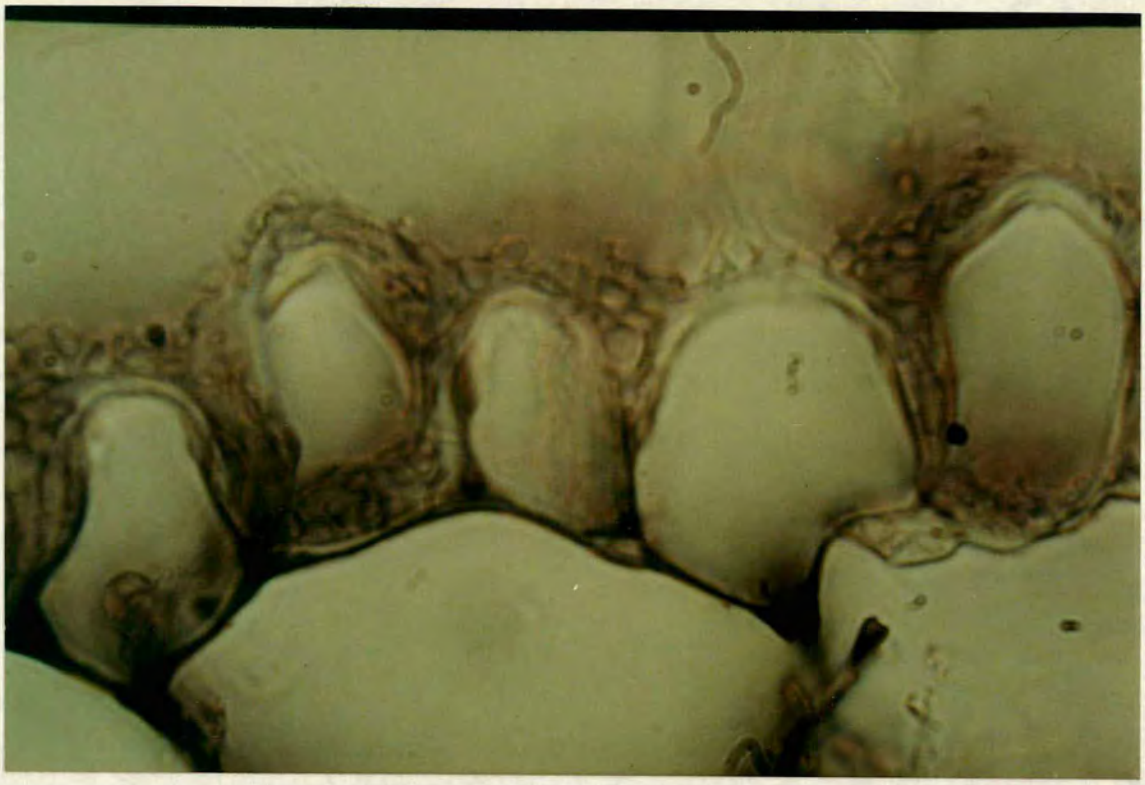
* All cell walls lignified.

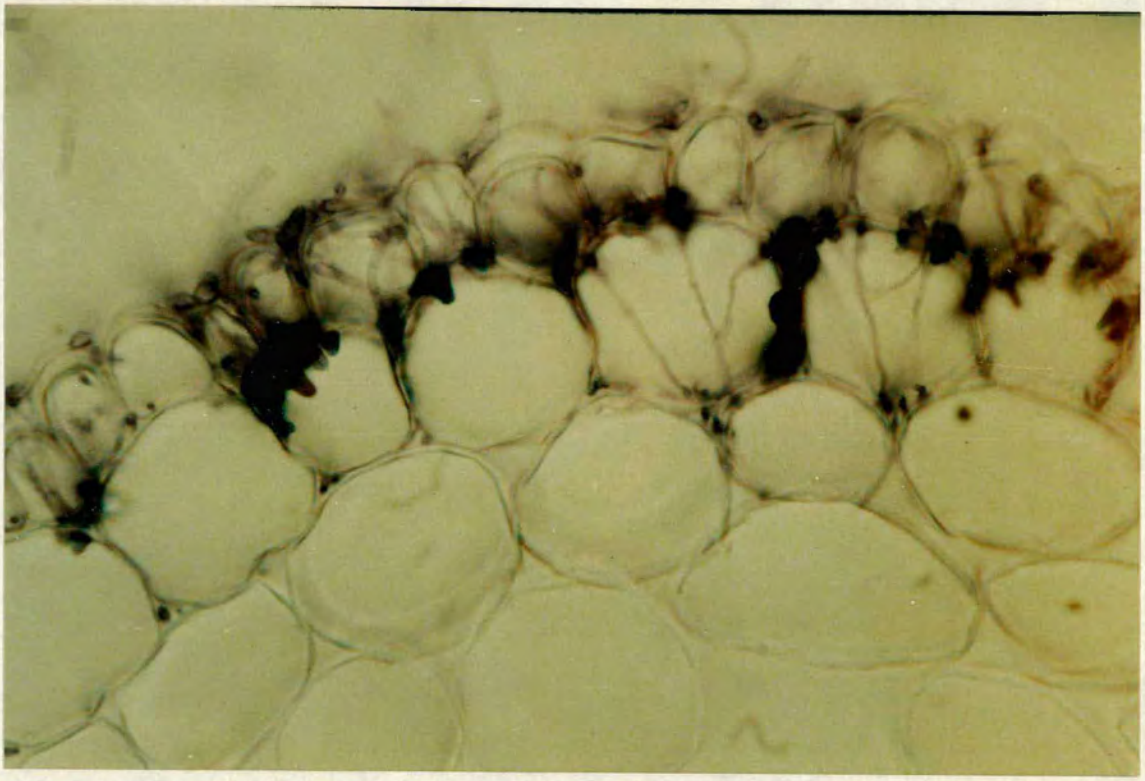
\- Not done.

Table 3.28. Occurence of yellow autofluorescence in walls of cells in wheat roots excised or attached to seedlings and uninoculated (controls) or inoculated with *M. bolleyi* or *P. lycopersici* (agar blocks or spores), then sampled at different times.

Cell layer	Roots attached							Roots detached						
	Control Day			Agar Day				spores Day				Control Day			Agar Day				Spores Day			
	1	3	5	1	3	5	7	1	3	5	7	1	3	5	1	3	5	7	1	3	5	7
												M. bolleyi										
1	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	+	*	*	*
2	*	+	*	*	*	*	*	+	*	*	*	+	*	*	*	*	*	*	+	*	*	*
3	0	+	+	+	+	+	*	0	+	*	*	0	+	*	+	+	*	*	0	0	*	*
4	0	+	+	0	+	+	*	0	+	*	*	0	+	*	0	0	*	*	0	0	*	*
5	0	0	+	0	+	+	*	0	0	*	*	0	+	+	0	0	+	*	0	0	*	*
6	0	0	+	0	+	+	*	0	0	*	*	0	+	+	+	0	+	*	0	0	*	*
7	+	*	+	0	+	+	*	0	*	*	*	+	*	*	*	*	+	*	+	+	*	*
												P. lycopersici										
1	+	*	*	*	*	*	-	+	*	*	-	+	+	*	+	*	*	-	+	+	*	-
2	+	*	*	*	*	*	-	+	*	*	-	*	*	*	+	*	*	-	+	+	*	-
3	0	+	0	*	*	*	-	0	+	+	-	0	+	+	+	*	*	-	0	0	+	-
4	0	+	0	0	+	*	-	0	0	+	-	0	0	+	+	*	*	-	0	0	0	-
5	0	0	0	0	+	*	-	0	0	+	-	0	0	+	+	*	*	-	0	0	0	-
6	0	0	0	0	*	*	-	0	0	+	-	+	0	+	+	*	*	-	0	0	0	-
7	0	+	+	+	*	*	-	0	+	+	-	*	+	*	+	*	*	-	0	*	+	-

0 No yellow autofluorescence observed.
+ Some cell walls were autofluorescing.
* Most cell walls were autofluorescing.
- Not done.

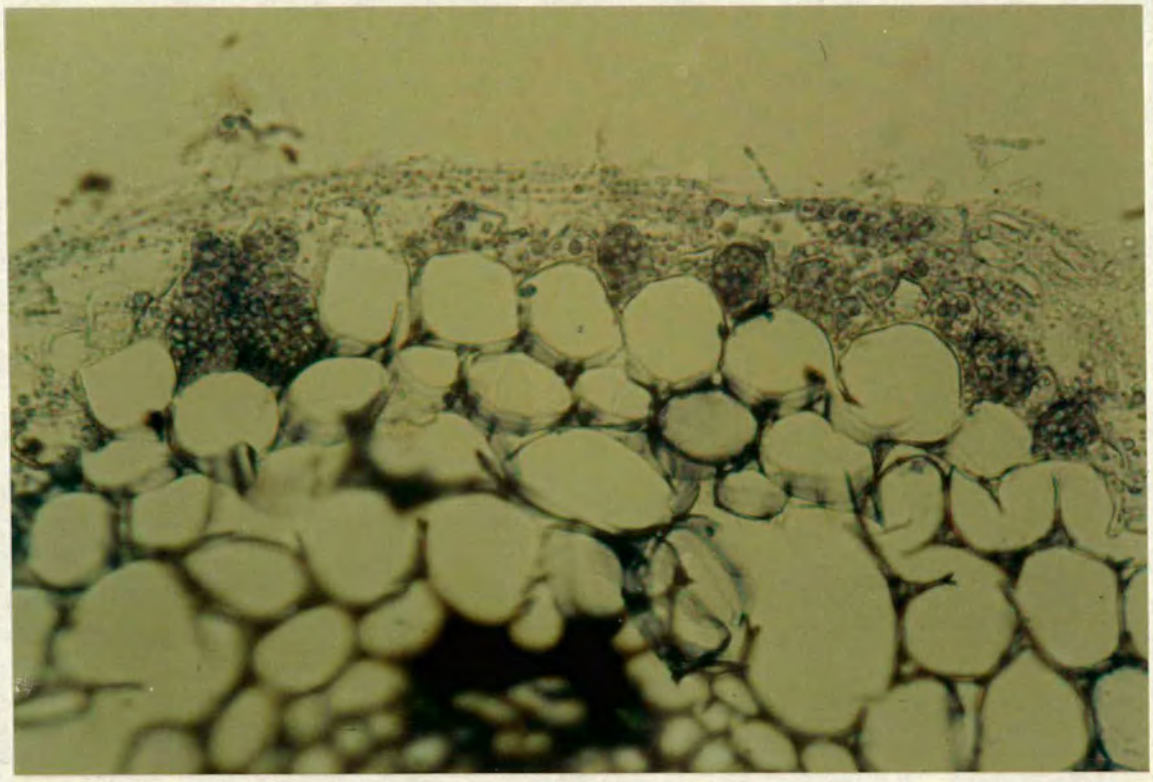


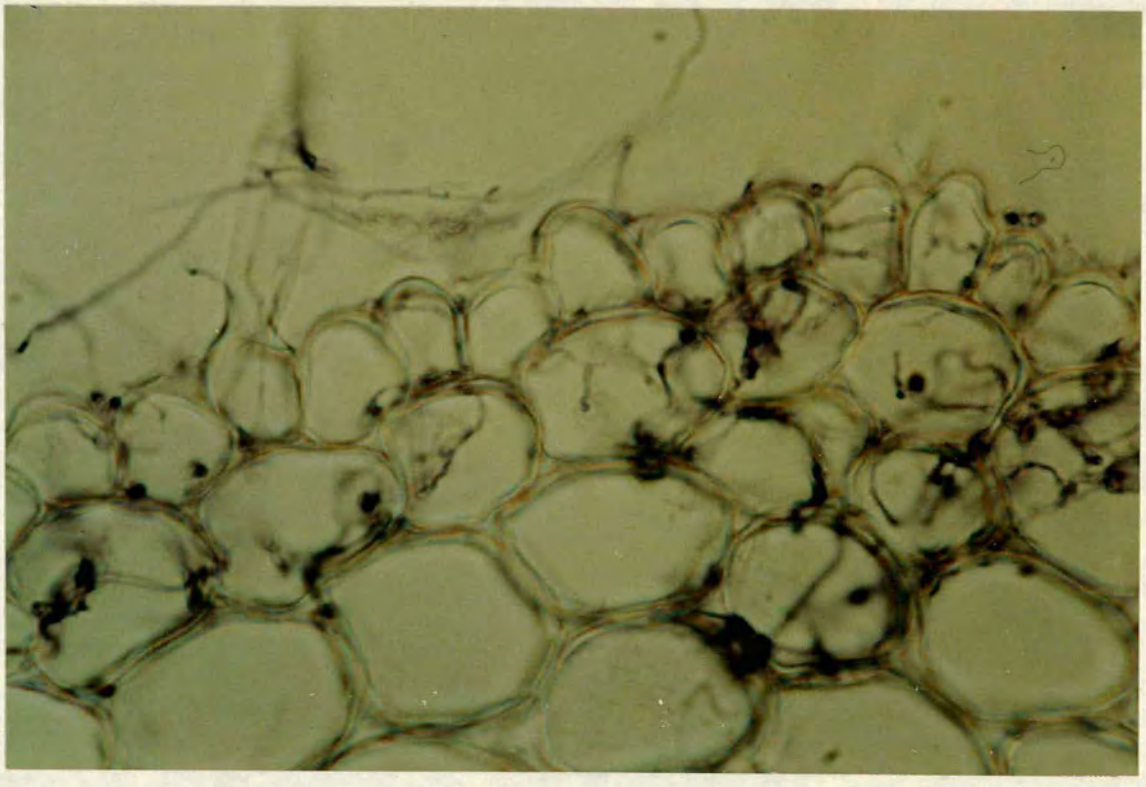


Figures 3.21, & 3.22. Transverse sections of wheat root attached to seedlings, stained with trypan blue to reveal fungal hyphae.

3.21. Two days after inoculation with *M. bolleyi*, showing extensive intercellular hyphae resembling the Hartig net of an ectomycorrhiza.

3.22. Four days after inoculation with *P. lycopersici*, showing predominantly intracellular penetration and the development of papillae in the hypodermis (cf Fig. 3.30).

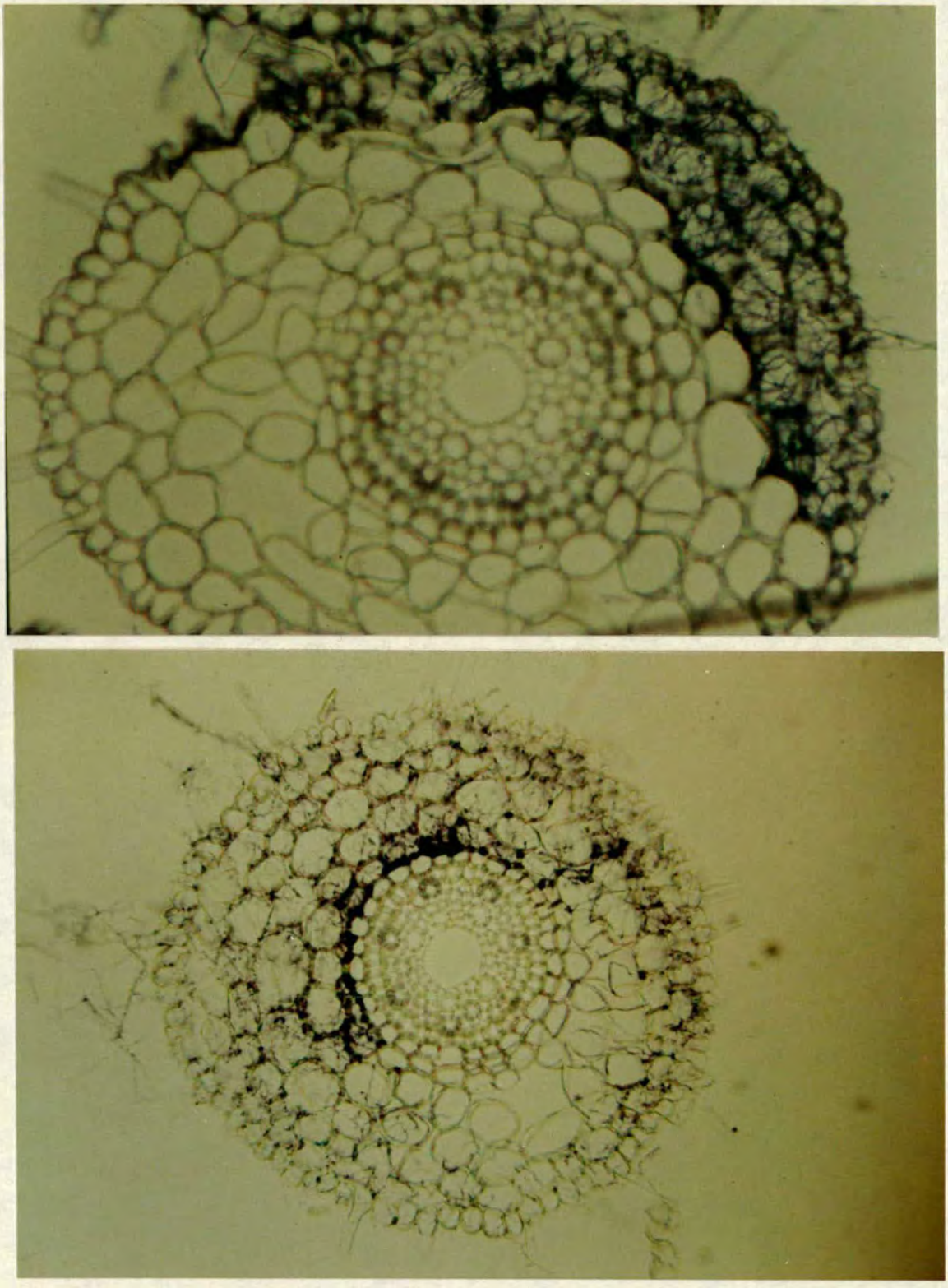




Figures 3.23, & 3.24. Transverse sections of wheat roots attached to seedlings, stained with trypan blue to reveal fungal hyphae.

3.23. Two days after inoculation with *M. bolleyi*, showing extensive growth of the fungus on the root surface and in the root epidermis, and largely intercellular penetration deeper into the cortex.

3.24. Two days after inoculation with *P. lycopersici*, showing predominantly intracellular penetration by the fungus from limited surface mycelia.



Figures 3.25, & 3.26. Transverse sections of wheat roots inoculated with *P. lycopersici* and stained with trypan blue.

3.25. Attached root 1 day after inoculation, showing unilateral invasion of up to 4 cell layers by the fungus. At the inner face of the invasion the host cells showed intense lignification reactions when treated with phloroglucinol-HCl.

3.26. Detached root 4 days after inoculation, showing extensive invasion up to the outer face of the endodermis.

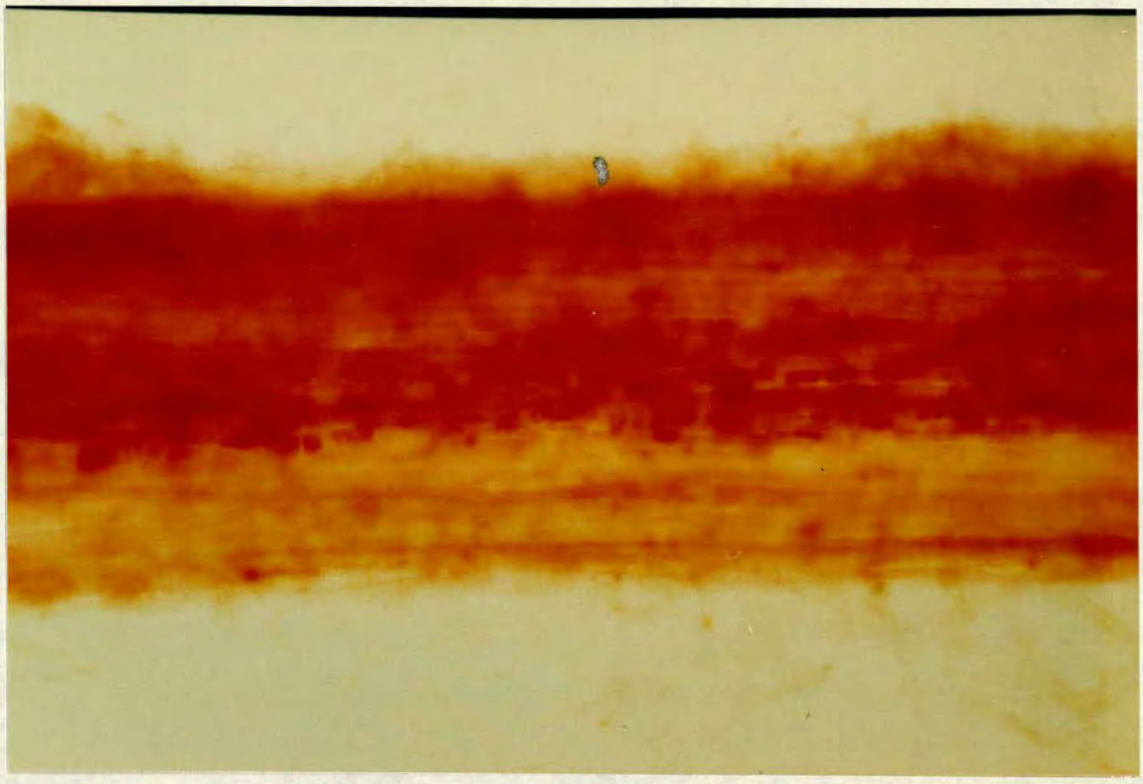


Fig. 3.27. Tomato root piece inoculated with *P. lycopersici* 24h previously, showing restriction of plasmolysable cells to the inner cortex.

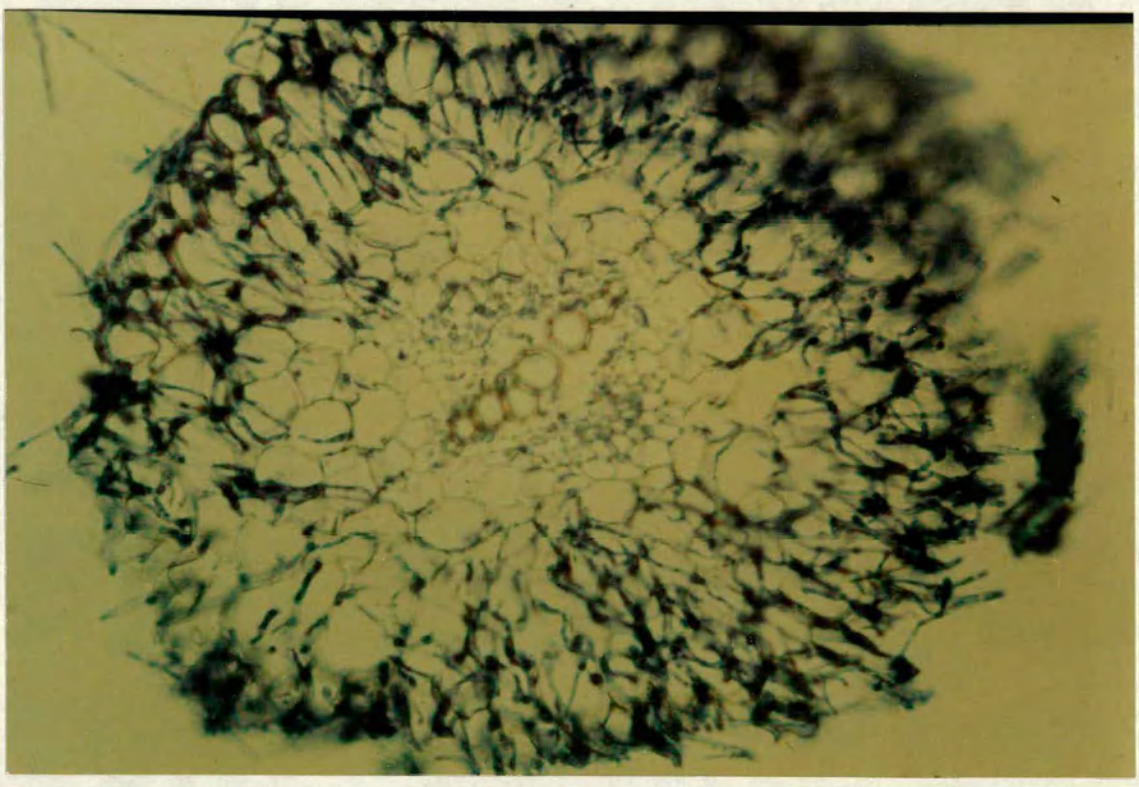
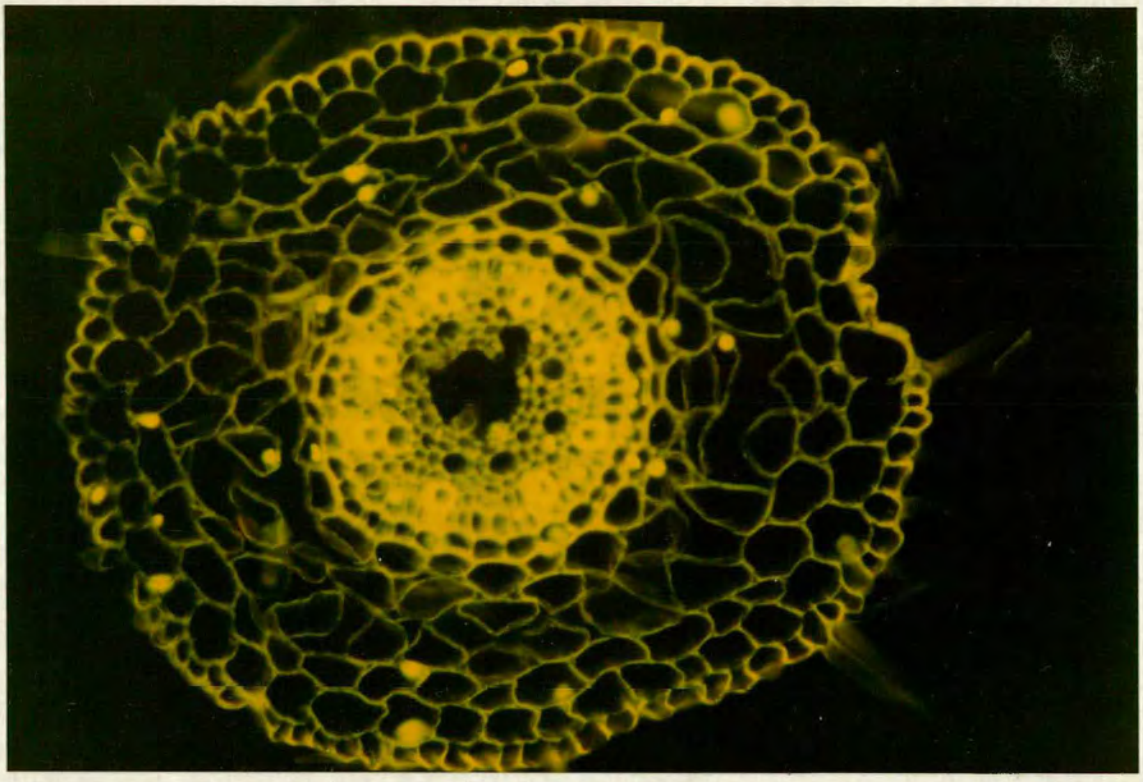
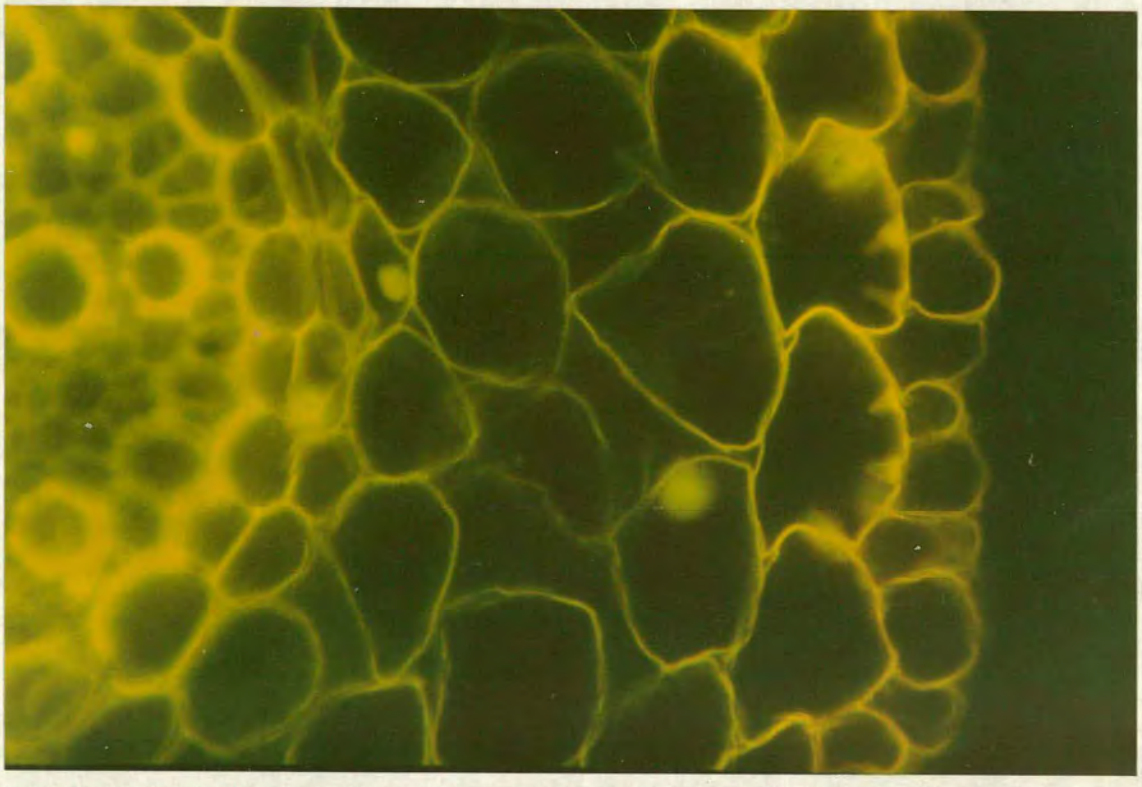


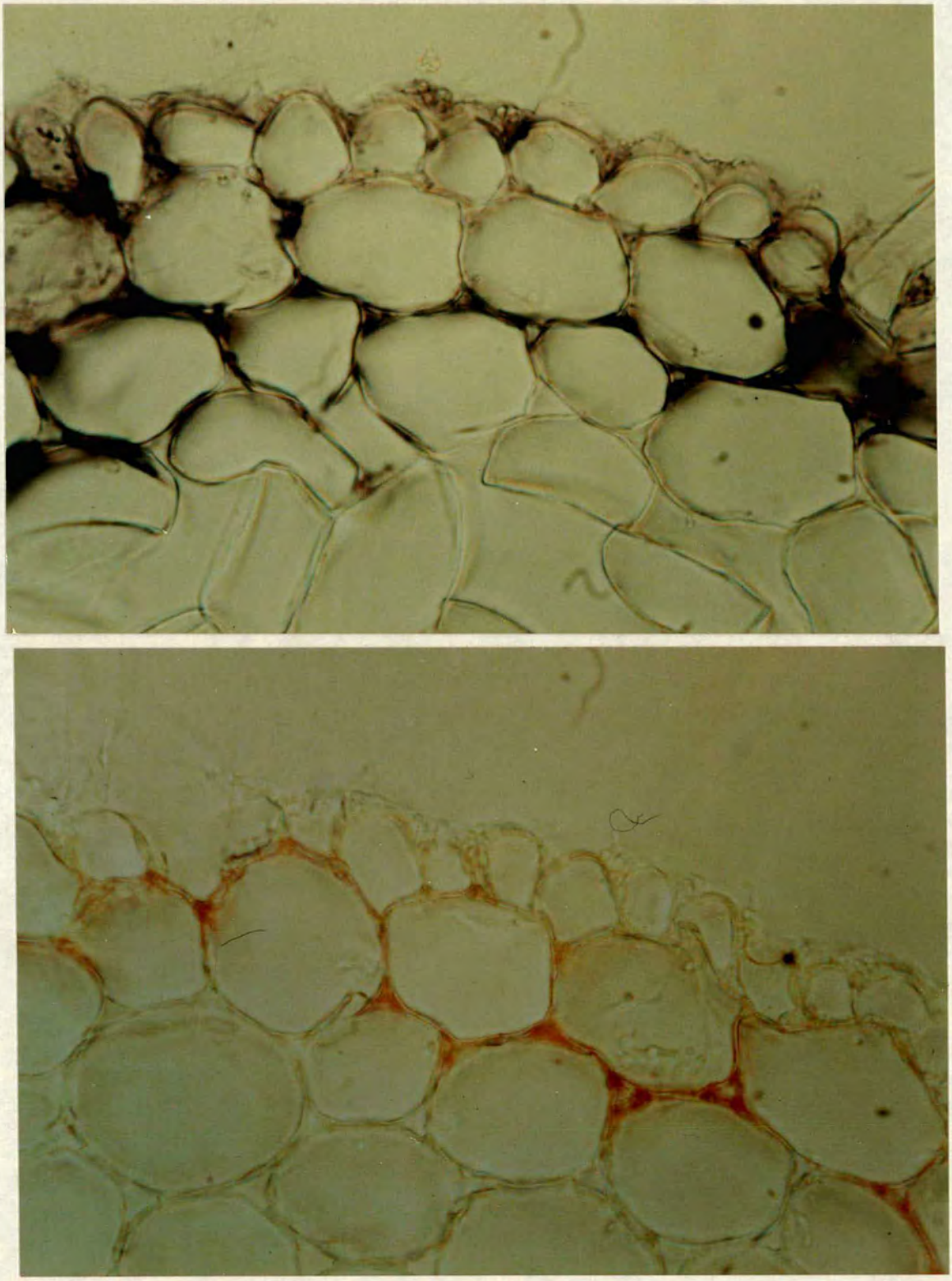
Fig. 3.28. Equivalent tomato root 24h after inoculation with *P. lycopersici* and stained with trypan blue to reveal extent of fungal invasion of the cortex.



Figures 3.29. AO staining of uninoculated 1-day old wheat root piece; distribution of nuclei in all cortical cell layers (v. thin section prepared for photograph, with fewer nuclei than would be seen in thicker sections).



3.30. Root attached to a seedling and inoculated with *P. lycopersici* 5 days earlier, showing nuclei in third and sixth cortical cell layers, and papillae formed in response to invasion in hypodermis.



Figures 3.31, & 3.32. Transverse sections of wheat roots inoculated with *M. bolleyi* 2 days previously.

2.31. Stained with trypan blue to reveal intercellular fungal hyphae.

2.32. Equivalent root treated with phloroglucinol-HCL to reveal intense lignification reactions in the hypodermis.

(Figures 3.22, 3.30). The lignitubers seen in roots inoculated with *M. bolleyi* were always smaller and in fewer numbers.

Yellow autofluorescence of roots also was enhanced by agar inocula of *P. lycopersici* (Table 3.28) but not markedly by spore inocula.

3.12. RCD in roots of wheat, barley, rye and oat, attached or detached from seedlings, and effects of inoculation with *M. bolleyi* and *Gaeumannomyces graminis* var *tritici*

This experiment was designed for a comparative study of RCD in the four main economic cereals of temperate zones, wheat, barley, rye and oats, and the effects of root excision in the presence of a weak parasite -*M. bolleyi*-and a pathogen -*Gaeumannomyces graminis* var *tritici* (*Ggt*).

Seedlings grown in Petri dishes were used to provide attached or detached roots as described in Section 3.12. The inocula of *M. bolleyi* were also as described in section 3.12 (5x15 mm blocks from the margins of colonies on PDA). For *Ggt* the inocula were either agar blocks from the margins of 6 day old colonies on PDA or, for a lower challenge inoculum, agar blocks from 8 day old colonies on water agar (WA), which had been started from a 1 cm disk cut from a colony on PDA. Strain BG1 of *Ggt* was used in this experiment.

The roots were sampled after 0, 2, 4, 6, and 8 days, five roots per treatment. A 10 day sampling also was used for attached oat roots. The roots were sectioned and assessed for presence of nuclei, hyphae, cell wall lignification and autofluorescence as described in Section 2.6.

3.12.1 Results

3.12.1.1. Comparison of RCD between the four cereals in roots attached and detached from seedlings

Figures 3.33 - 3.36 show the presence of nuclei in each cell layer, in the absence of inoculum or in the presence of *M. bolleyi* or *Ggt*, in attached or detached roots of wheat, barley, rye and oat respectively.

In order to enable comparisons between the four cereals, the mean numbers of nuclei present in each cell layer (average of 10 sections) are presented as percentages, taking as 100 the number of nuclei present in the same layer of young roots (day 0, mean of 20 sections).

For all cereals, roots attached to the seedlings had less RCD than the detached roots in uninoculated controls and across the range of inoculation treatments. For the attached control roots, wheat showed slightly more RCD than barley and rye, but all three cereals showed the same pattern of cell death - the numbers of nuclei in each cortical cell layer tended to decrease with time and decreased progressively from the outer cell layers inwards. Oats showed a different pattern of RCD, with a conspicuous loss of nuclei from the middle cortical cell layers with time but with nuclei tending to persist in the epidermis and, to a lesser degree in the hypodermis.

Detached oat roots showed the fastest RCD among all four cereals; there were few living cells in any cortical cell layer after six days and none after 8 days. Again nuclei persisted in the epidermis more than in the inner cell layers but this effect disappeared after the 2 day sampling. The other three cereals showed small and non-significant differences in RCD in detached roots.

3.12.1.2. Effects of inoculation with *M. bolleyi*

The presence of *M. bolleyi* had very little or no effect on RCD in attached roots of any cereal but it significantly increased the amount of RCD in detached roots of all four cereals (Appendices 4-7). The effect was more prominent in barley, rye and oat than in wheat, as seen particularly at the 4 day sampling time. But by 6 days *M. bolleyi* had killed all cell layers in the detached roots of all four cereals.

3.12.1.3. Effects of inoculation with *Gaeumannomyces graminis* var *tritici*

Both types of inoculum of *Ggt* significantly reduced cell viability in both attached and detached roots of the cereals (Appendices 4-7). Overall, PDA inocula had more effect than WA inocula, except on oats where there was little difference between them. Also, the effects of inoculation were more intense in detached roots than in attached roots of all cereals, although interpretation of this is complicated by the faster rate of RCD in detached compared with attached control (uninoculated) roots. The most interesting feature, however, was the effect of *Ggt* on the different cereals.

Wheat roots, attached or detached from seedlings, were severely and rapidly damaged by *Ggt* Barley was damaged to a similar degree. Rye was damaged to a somewhat lesser degree than either wheat or barley, especially in roots attached to seedlings. Oats were damaged least, such that they retained at least some living cell layers at the 8 day sampling in attached roots and had some nuclei in all cortical cell layers of detached roots at the 4 day sampling. Thus, for attached roots the cereals could be ranked in an order of

decreasing resistance as follows: oats then rye then barley and wheat, with little difference between the last two cereals. For detached roots, oats showed more resistance than rye, and wheat and barley were the most succeptible with, again, little difference between them.

3.12.1.4. Penetration by *M. bolleyi* and *Ggt*

As shown in Tables 3.29 *M. bolleyi* penetrated rapidly within 2 days the first one or two layers of the cortex of wheat, barley and rye attached to seedlings but made little further progress up to the 8 day sampling. In all these cereals, however, intercellular hyphae were seen deeper in the cortex than were intracellular hyphae, and their depth within the cortex increased with time.

In detached roots of wheat, barley and rye (Table 3.30), *M. bolleyi* also penetrated the outer one or two cell layers in the first 2 days; it made little or no further progress in the next 2 days, but by the sixth day it had penetrated further and intercellular hyphae had reached the endodermis.

Oat roots, both attached or detached, were penetrated poorly by *M. bolleyi*; only the epidermis or hypodermis was penetrated after 6 days in detached roots and after 10 days in roots attached to seedlings, although the fungus was also seen in the vascular tissues of detached roots, evidently because it invaded these from the cut ends.

From both WA and PDA inocula *Ggt* penetrated rapidly and deeply the cortex of wheat, barley and rye, soon reaching the stelar tissues of both attached and detached roots (Tables 3.31 - 3.34). It penetrated much more slowly into oat roots, whether they were attached to or detached from the seedlings. However it grew into the

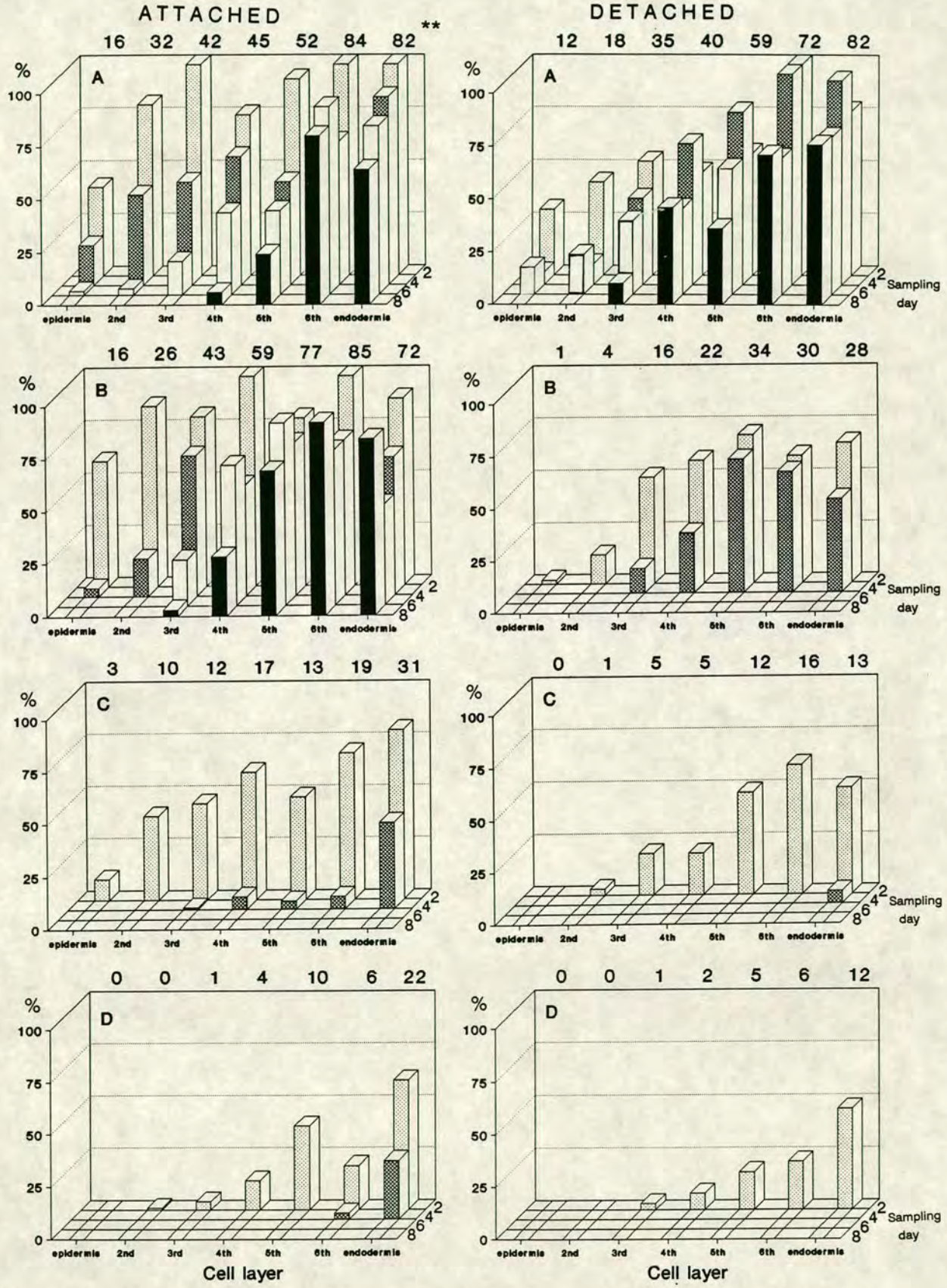
vascular system through the cut ends of detached oat roots, precluding meaningful assessments after the 6 day sampling. In the earliest (2 day) sampling, rye was evidently more resistant to penetration than were wheat and barley. This difference was significant in all but the combination of PDA inoculum and attached roots (Table 3.31) but it was not evident by the 4 day sampling in any combination, presumably because the pathogen overwhelmed the resistance of rye from the high inoculum level that was applied.

3.12.1.5. Host responses to invasion

M. bolleyi induced lignification in all cell layers of attached roots of wheat and rye (Table 3.35); detached roots showed the same reactions but to a lesser degree. Barley and oat roots showed little evidence of lignification responses except in the epidermis and hypodermis. The responses of the cereals to challenge by *Ggt* were similar to those in the presence of *M. bolleyi*, but in attached roots of wheat and barley they were more rapid and more intense. Only the data for PDA inocula are shown in Table 3.35.

Yellow autofluorescence was seen throughout the cortex of attached wheat, barley and rye roots challenged by *M. bolleyi* (Table 3.36), but only in the epidermis and hypodermis of oat roots. Again *Ggt* elicited more rapid and intense reactions in attached roots of all cereals than did *M. bolleyi*. Essentially similar results were obtained from detached roots, except that *M. bolleyi* caused as rapid and intense reactions as did *Ggt*.

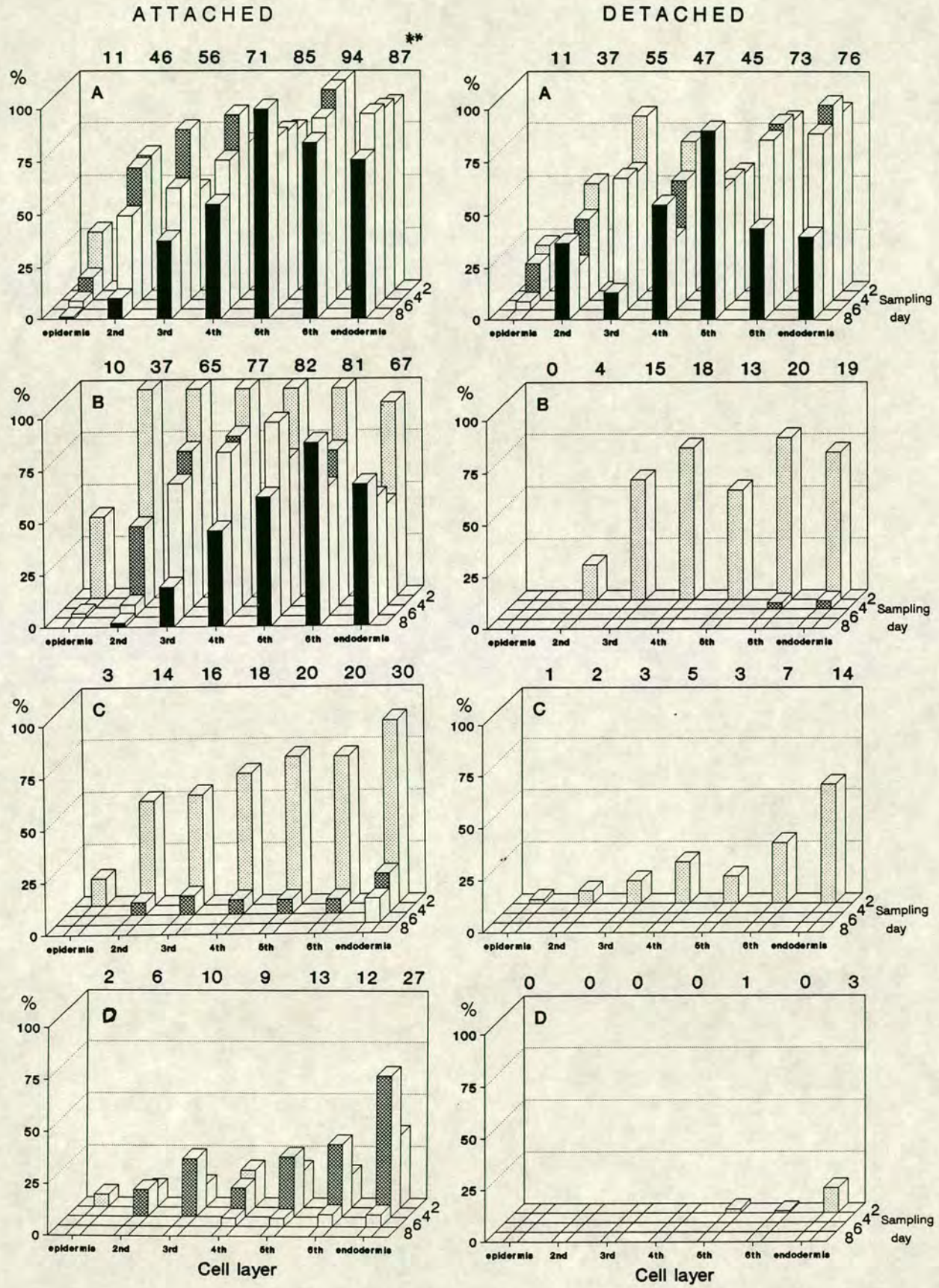
Figure 3.33. Wheat roots: percentage persistence of nuclei in each cortical cell layer of roots attached or detached from seedlings and non-inoculated (A) or inoculated with *M. bolleyi* (B); Ggt from WA (C) or Ggt from PDA (D); means of assessments of 10 sections per sample.*



* Percentages based on comparison with numbers of nuclei present in freshly excised roots (0-day sampling).

** Numbers above the histograms are means of values for all sampling days.

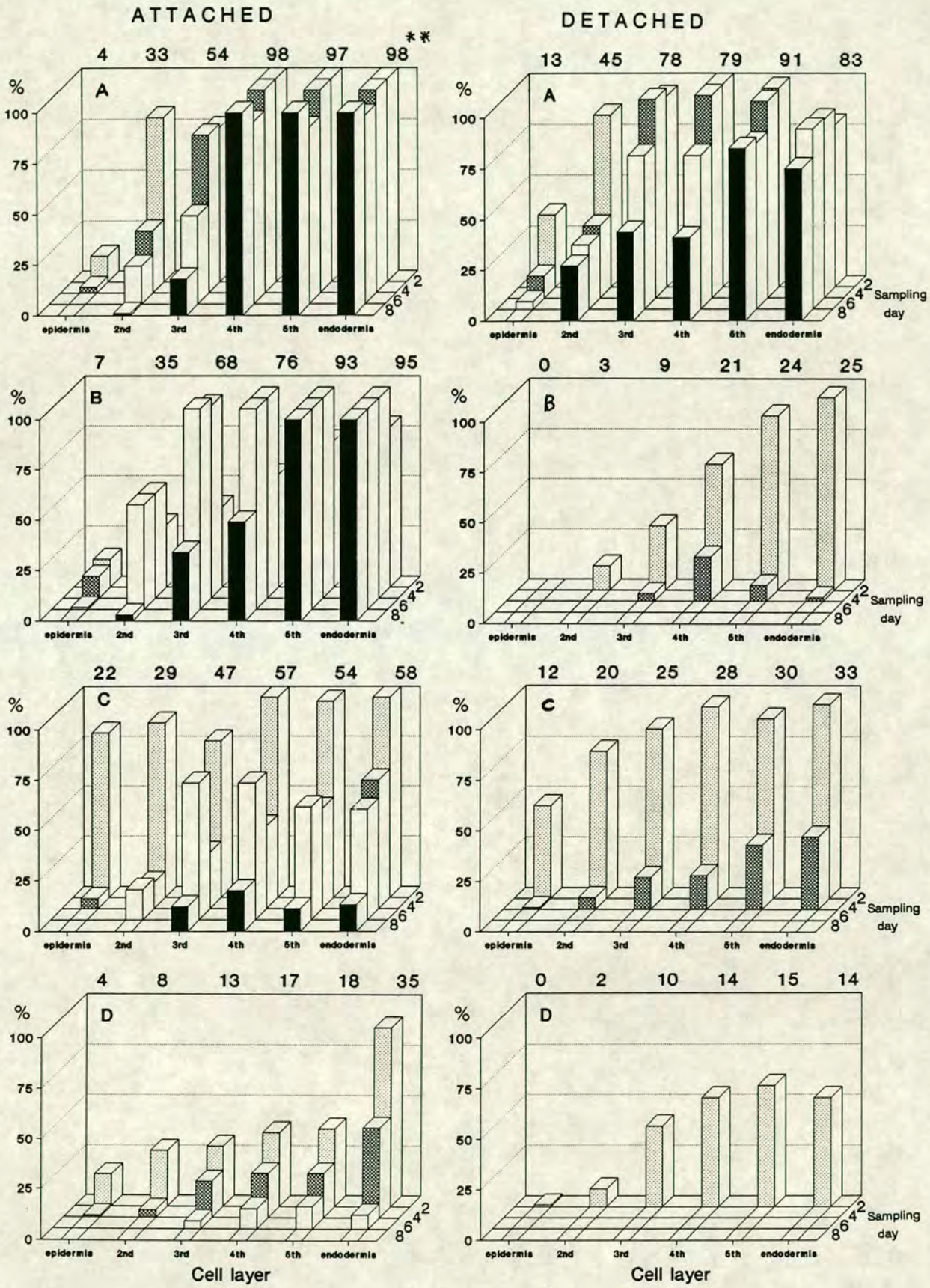
Figure 3.34. Barley roots: percentage persistence of nuclei in each cortical cell layer of roots attached or detached from seedlings and non-inoculated (A) or inoculated with *M. bolleyi* (B); Ggt from WA (C) or Ggt from PDA (D); means of assessments of 10 sections per sample.*



* Percentages based on comparison with numbers of nuclei present in freshly excised roots (0-day sampling).

** Numbers above the histograms are means of values for all sampling days.

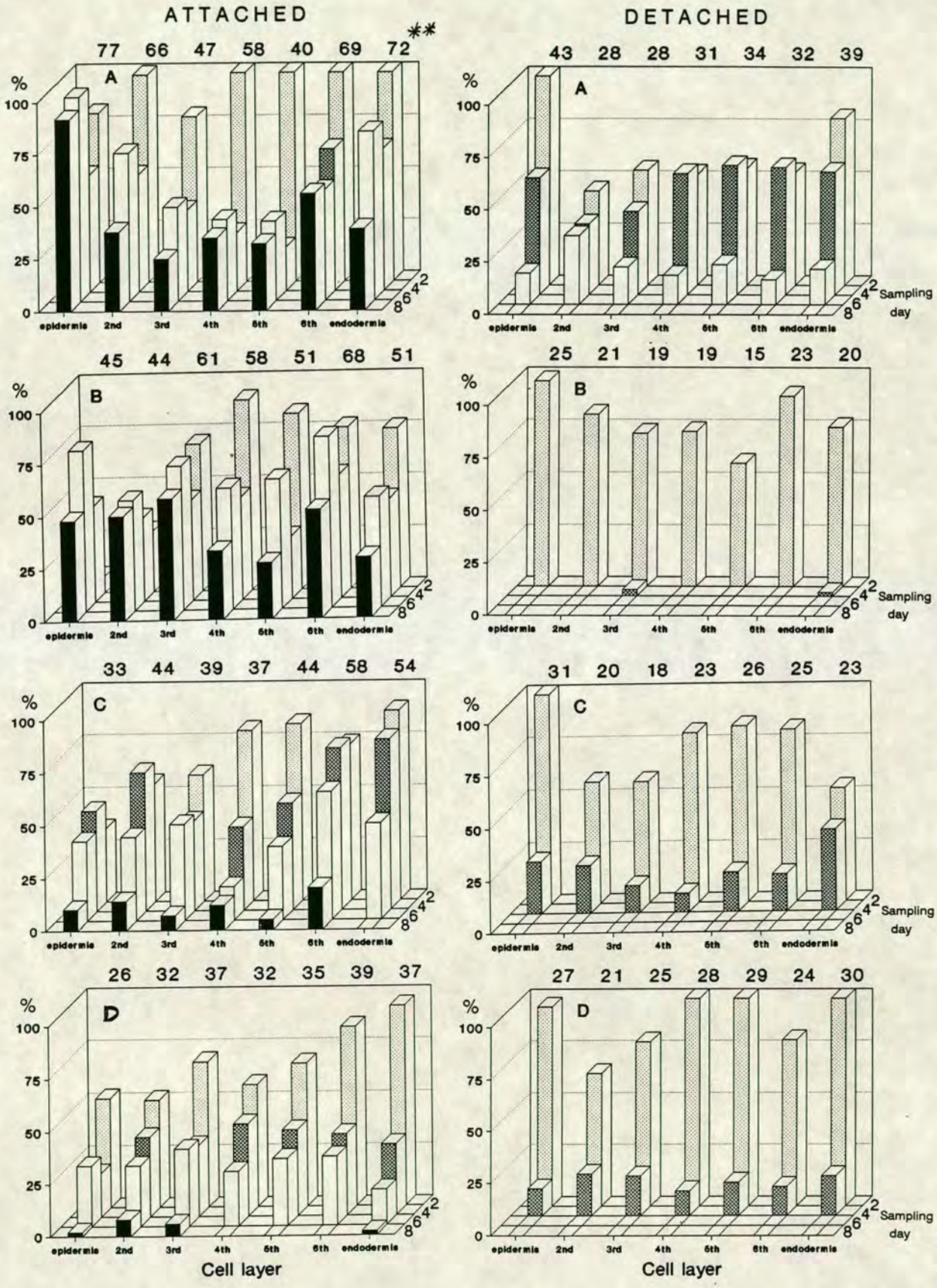
Figure 3.35. Rye roots: percentage persistence of nuclei in each cortical cell layer of roots attached or detached from seedlings and non-inoculated (A) or inoculated with *M. bolleyi* (B); Ggt from WA (C) or Ggt from PDA (D); means of assessments of 10 sections per sample. *



* Percentages based on comparison with numbers of nuclei present in freshly excised roots (0-day sampling).

** Numbers above the histograms are means of values for all sampling days.

Figure 3.36. Oat roots: percentage persistence of nuclei in each cortical cell layer of roots attached or detached from seedlings and non-inoculated (A) or inoculated with *M. bolleyi* (B); *Ggt* from WA (C) or *Ggt* from PDA (D); means of assessments of 10 sections per sample.*



* Percentages based on comparison with numbers of nuclei present in freshly excised roots (0-day sampling).

** Numbers above the histograms are means of values for all sampling days.

Table 3.29. Numbers* of cell layers penetrated by hyphae in roots of wheat, barley, rye and oat, attached to seedlings and inoculated with *Microdochium bolleyi*; assessed in transverse sections.

SAMPLING DAY		W H E A T	B A R L E Y	R Y E	O A T
2	Max.**	1.9±0.10	2.1±0.10	1.6±0.16	0.8±0.13
	Min.	0.6±0.16	0.8±0.13	0.2±0.13	0.0±0.00
	Average	1.2±0.09	1.3±0.04	1.0±0.13	0.2±0.05
4	Max.	1.9±0.10	2.8±0.20	2.5±0.27	0.9±0.10
	Min.	0.7±0.15	1.4±0.16	0.7±0.26	0.0±0.00
	Average	1.3±0.13	2.0±0.10	1.6±0.26	0.4±0.08
6	Max.	2.3±0.21 (3.1±0.23)	2.0±0.15 (2.7±0.21)	2.0±0.15	1.0±0.15
	Min.	1.3±0.15	1.2±0.13	0.8±0.20	0.0±0.00
	Average	1.8±0.08	1.7±0.30	1.4±0.17	0.4±0.09
8	Max.	2.4±0.16 (4.2±0.29)	2.3±0.21 (4.4±0.31)	2.3±0.21 (3.2±0.49)	1.2±0.13
	Min.	1.3±0.15	1.1±0.10	1.9±0.18	0.3±0.15
	Average	1.8±0.09	1.6±0.10	2.2±0.17	0.9±0.05
10	Max.				1.0±0.00
	Min.	N D	N D	N D	0.4±0.16
	Average				0.8±0.07

* Mean number of cell layers penetrated intracellularly ± S.E. of mean for 10 sections from each sample; in parentheses, mean depth (as cell layers) of intercellular penetration, where observed.

** Max. and min. = maximum and minimum depth of invasion around the circumference of each section; average = mean depth of penetration for 6 sectors around the circumference of the section.

Table 3.30. Numbers* of cell layers penetrated by hyphae in roots of wheat, barley, rye and oat, detached from seedlings and inoculated with *M. bolleyi*; assessed in transverse sections.

SAMPLING DAY		W H E A T	B A R L E Y	R Y E	O A T
2	Max.**	2.2±0.13	2.6±0.22 (2.7±0.21)	2.1±0.10 (2.6±0.27)	1.0±0.00
	Min.	1.0±0.26	0.8±0.29	0.9±0.28	0.1±0.10
	Average	1.7±0.13	1.7±0.17	1.5±0.45	0.5±0.09
4	Max.	2.2±0.13 (3.9±0.40)	2.7±0.26 a (4.6±0.31)	2.5±0.17 a (3.8±0.29)	1.6±0.43 a
	Min.	1.5±0.17	1.1±0.10	1.6±0.16	0.2±0.13
	Average	1.9±0.43	1.9±0.09	2.1±0.11	0.8±0.13
6	Max.	3.5±0.92 a (5.7±0.55)	4.0±0.87 a (5.8±0.74)	8.0±0.00	1.9±0.38 a
	Min.	3.0±1.01	3.3±0.96	8.0±0.00	0.3±0.15
	Average	3.3±0.96	3.5±0.92	8.0±0.00	1.0±0.19

* Mean number of cell layers penetrated intracellularly ± S.E. of mean for 10 sections from each sample; in parentheses, mean depth (as cell layers) of intercellular penetration, where observed.

** Max. and min. = maximum and minimum depth of invasion around the circumference of each section; average = mean depth of penetration for 6 sectors around the circumference of the section.

a Presence of hyphae in the vascular elements.

Table 3.31. Numbers* of cell layers penetrated by hyphae in roots of wheat, barley, rye and oat, attached to seedlings and inoculated with *Gaeumannomyces graminis* var *tritici*, WA-inocula; assessed in transverse sections.

SAMPLING DAY		W H E A T	B A R L E Y	R Y E	O A T
2	Max.**	4.6±0.52	4.4±0.62	2.5±0.67	0.2±0.13
	Min.	0.8±0.13	0.7±0.60	0.0±0.00	0.0±0.00
	Average	2.6±0.28	2.3±0.47	1.0±0.31	0.0±0.02
4	Max.	6.4±0.04	6.9±0.59	8.0±0.00	0.8±0.25
	Min.	4.6±0.60	6.1±0.81	5.4±0.77	0.0±0.00
	Average	5.6±0.00	6.5±0.67	7.0±0.28	0.2±0.08
6	Max.	9.0±0.00	9.0±0.00	7.7±0.76	2.1±0.46
	Min.	9.0±0.00	9.0±0.00	6.5±1.27	0.0±0.00
	Average	9.0±0.00	9.0±0.00	7.1±1.03	0.9±0.23
8	Max.			6.2±1.15	2.2±0.51
	Min.	N D	N D	4.2±1.32	0.0±0.00
	Average			5.1±1.15	1.0±0.29
10	Max.				4.7±0.84
	Min.	N D	N D		0.8±0.61
	Average				2.6±0.66

* Mean number of cell layers penetrated intracellularly ± S.E. of mean for 10 sections from each sample.

** Max. and min. = maximum and minimum depth of invasion around the circumference of each section; average = mean depth of penetration for 6 sectors around the circumference of the section.

Table 3.32. Numbers* of cell layers penetrated by hyphae in roots of wheat, barley, rye and oat, detached from seedlings and inoculated with *Gaeumannomyces graminis* var *tritici*, WA-inocula; assessed in transverse sections.

SAMPLING DAY		W H E A T	B A R L E Y	R Y E	O A T
2	Max.**	4.3±0.50	6.4±0.64	0.7±0.21	0.1±0.10
	Min.	0.6±0.16	2.0±0.89	0.0±0.00	0.0±0.00
	Average	2.2±0.07	4.7±0.77	1.2±0.07	0.0±0.00
4	Max.	7.9±0.38 a	8.6±0.40 a	4.9±0.06 a	1.0±0.26 a
	Min.	7.4±0.54	8.3±0.70	4.3±1.16	0.0±0.00
	Average	7.6±0.47	8.4±0.60	4.7±1.09	0.3±0.06
6	Max.			8.0±0.00 a	0.4±0.16 a
	Min.	N D	N D	8.0±0.00	0.0±0.00
	Average			8.0±0.00	0.1±0.04

* Mean numbe of cell layers penetrated intracellularly ± S.E. of mean for 10 sections from each sample.

** Max. and min. = maximum and minimum depth of invasion around the circumference of each section; average = mean depth of penetration for 6 sectors around the circumference of the section.

a Presence of hyphae in the vascular elements.

Table 3.33. Numbers* of cell layers penetrated by hyphae in roots of wheat, barley, rye and oat, attached to seedlings and inoculated with *Gaeumannomyces graminis* var *tritici*, PDA-inocula; assessed in transverse sections.

SAMPLING DAY		W H E A T	B A R L E Y	R Y E	O A T
2	Max.**	6.4±0.22	5.8±0.44	7.0±0.49	0.6±0.22
	Min.	2.9±0.41	3.5±0.85	2.6±0.93	0.0±0.00
	Average	5.0±0.29	4.9±0.53	4.9±0.63	0.2±0.08
4	Max.	9.0±0.00	7.4±0.56	7.8±0.40	0.6±0.16
	Min.	9.0±0.00	6.8±0.88	7.5±0.89	0.0±0.00
	Average	9.0±0.00	6.9±0.78	7.6±0.76	0.2±0.06
6	Max.	9.0±0.00	8.7±0.30	7.6±0.93	3.0±0.63
	Min.	8.8±0.20	8.1±0.89	7.5±1.00	0.2±0.13
	Average	8.9±0.07	8.5±0.52	7.6±0.96	1.4±0.33
8	Max.			8.0±0.00	5.8±0.20
	Min.	N D	N D	8.0±0.00	3.6±0.98
	Average			8.0±0.00	4.8±0.53
10	Max.				5.4±0.51
	Min.	N D	N D	N D	2.9±0.78
	Average				4.1±0.63

* Mean number of cell layers penetrated intracellularly ± S.E. of mean for 10 sections from each sample.

** Max. and min. = maximum and minimum depth of invasion around the circumference of each section; average = mean depth of penetration for 6 sectors around the circumference of the section.

Table 3.34. Numbers* of cell layers penetrated by hyphae in roots of wheat, barley, rye and oat, detached from seedlings and inoculated with *Gaeumannomyces graminis* var *tritici*, PDA-inocula; assessed in transverse sections.

SAMPLING DAY		W H E A T	B A R L E Y	R Y E	O A T
2	Max.**	6.0±0.56	8.4±0.31	5.2±0.93 (5.5±0.90)	1.0±0.37
	Min.	2.4±0.43	8.1±0.46	3.5±1.21	0.0±0.00
	Average	4.4±0.49	8.3±0.38	4.3±1.05	0.2±0.00
4	Max.	9.0±0.00 a	6.8±0.93 a (7.5±0.62)	8.0±0.00 a	0.1±0.10
	Min.	9.0±0.00	5.9±1.27	8.0±0.00	0.0±0.00
	Average	9.0±0.00	6.3±1.13	8.0±0.00	0.0±0.00
6	Max.			8.0±0.00 a	2.6±0.65 a
	Min.	N D	N D	8.0±0.00	0.0±0.00
	Average			8.0±0.00	1.0±0.30

* Mean number of cell layers penetrated intracellularly ± S.E. of mean for 10 sections from each sample; in parentheses, mean depth (as cell layers) of intercellular penetration, where observed.

** Max. and min. = maximum and minimum depth of invasion around the circumference of each section; average = mean depth of penetration for 6 sectors around the circumference of the section.

a Presence of hyphae in the vascular elements.

Table 3.35. Occurrence of lignification reactions (assessed with phloroglucinol-HCl) in walls of cells of roots of wheat, barley, oat and rye, inoculated with *M. bolleyi* (Mb) or *Gaeumannomyces graminis* var *tritici* (Ggt).

	Roots attached						Roots detached					
Cell	Control			Mb		Ggt	Control			Mb		Ggt
layer	Day			Day		Day	Day			Day		Day
	0	2 4 6		2 4 6		2 4 6	0	2 4 6		2 4 6		2 4 6
					Wheat							
1	0	0 0 +		+ 0 +		* * *	0	+ + +		+ + *		0 + -
2	0	0 0 +		* + +		* * *	0	+ 0 0		+ * *		+ * -
3	0	0 0 0		+ 0 +		* * *	0	0 0 0		0 0 +		0 + -
4	0	0 0 0		0 0 +		+ * *	0	0 0 0		0 0 0		0 + -
5	0	0 0 0		0 0 +		+ * *	0	0 0 0		0 0 0		0 + -
6	0	0 0 0		0 0 +		+ * *	0	0 0 0		0 0 0		0 + -
7	+	* + *		* + *		+ * *	0	+ * *		+ * *		+ + -
					Barley							
1	0	0 0 0		+ 0 0		0 0 0	0	0 0 +		+ + 0		+ + -
2	0	0 0 0		+ + 0		0 0 0	0	0 0 0		+ 0 0		0 * -
3	0	0 0 0		0 + 0		0 0 0	0	0 0 0		0 0 0		0 0 -
4	0	0 0 0		0 0 0		0 0 0	0	0 0 0		0 0 0		0 0 -
5	0	0 0 0		0 0 0		0 0 0	0	0 0 0		0 0 0		0 0 -
6	0	0 0 0		0 0 0		0 0 0	0	0 0 0		0 0 0		0 0 -
7	+	+ + +		* * +		+ + +	+	* * *		* * *		* * -
					Oat							
1	0	0 + 0		0 0 +		0 + +	0	+ 0 +		+ + +		+ + +
2	0	0 0 0		0 + 0		0 + 0	0	0 0 0		0 0 0		0 0 0
3	0	0 0 0		0 0 0		0 0 0	0	0 0 0		0 0 0		0 0 0
4	0	0 0 0		0 0 0		0 0 0	0	0 0 0		0 0 0		0 0 0
5	0	0 0 0		0 0 0		0 0 0	0	0 0 0		0 0 0		0 0 0
6	0	0 0 0		0 0 0		0 0 0	0	0 0 0		0 0 0		0 0 0
7	+	+ * *		+ * +		+ * +	+	* * *		* * *		* * *
					Rye							
1	0	0 0 0		0 + +		* * *	0	+ + +		+ + 0		+ + +
2	0	0 + 0		+ * +		* * *	0	0 0 0		0 + 0		+ 0 +
3	0	0 0 0		+ + +		* * *	0	0 0 0		0 + 0		+ 0 0
4	0	0 0 0		0 + +		* * *	0	0 0 0		0 0 0		0 0 0
5	0	0 0 0		0 0 0		* * *	0	0 0 0		0 0 0		0 0 0
6	+	+ * *		+ * *		* * *	+	* * *		* * +		* * *

0 No lignified cell walls.
\+ Some cell walls lignigied.
* All cell walls lignified.
\- Not done .

Table 3.36. Occurrence of yellow autofluorescence in walls of cells of roots attached to or detached from seedlings of wheat, barley, oat and rye, inoculated with *M. bolleyi* (Mb) or *Gaeumannomyces graminis* var *tritici* (Ggt).

	Roots attached			Roots detached		
Cell	Control	M b	Ggt	Control	M b	Ggt
layer	Day	Day	Day	Day	Day	Day
	0 2 4 6	2 4 6	2 4 6	0 2 4 6	2 4 6	2 4 6
			Wheat			
1	* * + +	* + *	* * *	* * * *	* + *	* * -
2	* * * *	* * *	* * *	* + + *	* + *	* * -
3	0 0 + 0	+ + *	* * *	0 0 0 0	0 + *	+ * -
4	0 0 + 0	0 + *	+ * *	0 0 0 0	0 + *	+ * -
5	0 0 + 0	0 + *	+ * *	0 0 0 0	0 + *	0 + -
6	0 0 + *	0 * *	+ * *	0 0 0 0	+ + *	0 + -
7	* + + *	+ * *	+ * *	* + + +	+ 0 *	+ + -
			Barley			
1	* * + *	* * *	* * *	* * * *	* * *	+ * -
2	* + + *	* * *	* * *	* * * +	* * *	0 * -
3	0 0 + *	* * *	* + *	0 0 + 0	* + *	0 * -
4	0 0 + +	+ + +	* + *	0 0 0 0	* + *	0 * -
5	0 0 0 +	+ + +	* + *	0 0 0 0	* + *	0 + -
6	0 0 0 +	+ + +	* + *	0 0 0 0	* + *	0 + -
7	+ + 0 *	+ + +	* + *	* + + +	* + *	0 + -
			Oat			
1	* * * *	* * *	* * *	* * * *	* * *	* * *
2	0 0 * +	+ + +	+ + +	0 0 + +	+ + *	+ + *
3	0 0 + +	0 0 0	0 0 +	0 0 + +	0 + 0	0 0 0
4	0 0 + +	0 0 0	0 0 +	0 0 0 0	0 + 0	0 0 0
5	0 0 + 0	0 0 0	0 0 +	0 0 0 0	0 + 0	0 0 0
6	0 0 0 0	0 0 0	0 0 +	0 0 0 +	0 + 0	0 0 0
7	0 0 + 0	0 0 0	0 0 +	0 + 0 +	0 + 0	0 + 0
			Rye			
1	* + * *	+ * *	+ * *	* * * *	* * *	* * *
2	0 * * *	* * *	* * *	0 + * *	* * *	* * *
3	0 0 0 +	+ + +	+ + *	0 0 0 0	+ * *	+ * *
4	0 0 0 +	0 + 0	+ + *	0 0 0 0	+ * *	+ * *
5	0 0 0 0	0 + 0	+ + *	0 0 0 0	+ * *	+ * *
6	+ 0 0 *	0 + *	+ + *	* * + *	* * *	+ * *

0 No yellow autofluorescence in cell walls.
\+ Some cells showed yellow autofluorescence.
* All cells showed yellow autofluorescence
\- Not done.

3.13. Discussion

Much of the work to date on early senescence of roots has been done by root pathologists concerned with understanding of the behaviour of pathogenic fungi and their potential biocontrol agents. The main exceptions involve, first, studies on shedding of root cortical tissues associated with secondary development of roots of dicotyledonous plants and, second, studies on the development of aerenchyma in oxygen-limiting conditions (Head, 1973; Drew *et al.*, 1981) Neither of these latter processes is directly equivalent to the early progressive cortical senescence in roots of cereals (Henry & Deacon, 1981).

Although there exist many methods for assessment of the viability of plant cells, a limited range of them have been used to assess cell viability in roots. The main ones involved nuclear staining with Feulgen reagent, AO, (Holden, 1975; Henry & Deacon 1981, Smiley & Giblin, 1986) or trypan blue (Brown & Hornby, 1987).

Published reports show that these methods give broadly equivalent results for cortices of cereals, as confirmed here (Fig. 3.1). The validity of AO staining for assessment of cell viability is discussed by Henry & Deacon (1981). But the use of AO has not, in general, revealed an equivalent progressive early senescence of the cortex of dicotyledonous plants, with the possible exception of an unsubstantiated report of senescence in roots of rape but not in subterranean clover (MacLeod *et al.*, 1986). There are however reports indirectly suggesting that root cortices in dicotyledonous plants may exhibit senescence -for example peanut plants growing in sterile conditions are reported to show extensive sloughing of cortical cells (Griffin *et al.*, 1976).

One of the aims of the work in this section was to compare assessment methods, particularly as they relate to cell death in different plants and for roots treated in different ways that might delay or enhance the rate of cell death. This was also important in an attempt to identify the earlier stages of cell senescence, which are likely to be most relevant to invasion by specialised root-infecting fungi, enabling them to colonise incipiently senescing cells before these can be colonised by soil saprophytes. The results in this section confirmed the validity of nuclear staining with AO as a criterion of cell viability in wheat cortices - both for roots with natural senescence (Fig. 3.1) and for those in which RCD was accelerated due to the presence of *M. bolleyi* (Fig. 3.12).

This method based on nuclear staining cannot, however, be used in tomato roots, since dead roots have stainable nuclei. MacLeod *et al.* (1986), reported that 25% of root cortical cells in rape seedlings, treated with AO were anucleate and thus were dead. Given the results for AO staining of tomato roots in Section 3.2, these findings should be reexamined using also other cytological methods for assessment of viability.

The neutral red stain combined with plasmolysis was satisfactory for both cereal and tomato roots. It can be considered as a reliable test since it assesses the integrity of cell membranes, a fundamental attribute of living cells. It must however be mentioned that cells with a disrupted plasmalemma but an intact tonoplast still can appear to plasmolyse (Robards, 1970) but this is unlikely to have happened in the roots examined.

Fluorecein diacetate gave poor results and can have only a limited use in detection of living or dead root cells, particularly

as it does not permit accurate quantitative assessments of viability.

The above-mentioned cytological tests enabled distinctions between living and dead or dying cells, but were unsuitable for determining the point where a cell is in the process of reversible and later irreversible changes leading to senescence. These phases are of particular interest to root pathologists and rhizosphere biologists since it is expected that members of the root microflora might differ in their ability to invade partially senescent cells and tissues. For this reason, other methods also were tested.

In the few reports of changes in the amount of DNA during the senescence of plant tissues (i.e. in leaves and cotyledons, reviewed by Brady (1988) it seems that the amount of DNA is slightly reduced during the senescence process. The loss of stainable nuclei in senescent cortical cells in cereal roots indicates major changes in the DNA content of these cells. It is not known, however, if this disappearance of nuclei occurs early or only in late stages of the senescence process.

The cytochemical methods used here (Section 3.2) showed differences in the distribution of DNAases between cortical cells in wheat roots and also differences in distribution of acid phosphatases within cells. A diffuse state of acid phosphatases observed in cells of embryos (Berjaak & Villiers, 1970) or differentiating xylem vessels (Gahan & Maple, 1966) was associated with senescence and death. Similar differences observed here in wheat root cortices perhaps could be used to assess early phases of senescence, but more accurate localization of these enzymes within the cells is needed in order to explore this possibility. The freeze microtome or free-hand sectioning used here did not give good enough

preservation of the internal structure of the highly vacuolated cortical cells to make these methods useful. But it is notable that there were no obvious differences in enzyme distribution or activities in cells of tomato root cortices, even 5 days after excision of the roots. This is perhaps an additional indication that either these roots did not senesce or they did so in a different way from that in cereals.

The main findings from the evaluation of methods for assessment of senescence in this section of the thesis were, therefore, as follows. AO is suitable for use with cereals but not with tomato. AO seems suitable for cereal roots treated in different ways because it correlates with NR-plasmolysis in all cases. Other methods are either not sensitive enough or not as easy to use routinely as are AO or NR-Plasmolysis. In addition, AO is valuable because it can be used on fixed roots and is thus suitable for use in large experiments which can be assessed at leisure.

The second major aim of the work in this section was to study the practicability of using root pieces in studies of host-parasite interactions. The potential advantage of this is that RCD might be manipulated by externally applied factors but independently of the effects of these factors on physiology of the rest of the plant, which might have indirect or consequential effects on RCD in particular root regions (Lewis & Deacon, 1982).

Ideally such studies should be done in liquid culture media, but this is not practicable for inoculations of roots with fungi, the underlying reason for exploring the phenomenon of RCD in this thesis. So all studies were done on agar media. An early finding was that tomato roots were so fragile that they could not be removed

from agar, so they were left on the original plates on which aseptic seedlings had been grown and the rest of the seedling was removed.

As reported previously (Kirk, 1984; Deacon & Lewis, 1986; Gillespie, 1986; Gillespie & Deacon, 1988) the pattern of RCD in excised wheat root pieces was exactly the same as that in whole, attached roots (Henry & Deacon, 1981), starting from the outer cortex and progressing inwards. There is also a clear pattern of senescence along root pieces, the older parts showing more RCD at any one time. These results justified the use of root pieces in studies of RCD.

Weste (1972) and others have studied host-parasite interactions by inoculating roots of sterile seedlings growing in closed Petri dishes. In the work described here both root pieces and roots attached to seedlings were used for comparison.

One major finding (Sections 3.2 & 3.3) was that the tomato root cortex does not senesce in the manner seen in wheat root cortices. When tomato root pieces were maintained on medium without sugar, they showed progressive death of cells begining in the epidermis and then extending rapidly inwards, but this occurred only late during incubation and once it started was very fast, suggesting a collapse of root function rather than a progressive, programmed senescence as in wheat. There was also a high degree of variability in the longevity of individual root pieces. In contrast, tomato root pieces showed prolonged viability when maintained on agar media amended with even low levels of sucrose, whereas cereal roots in equivalent conditions still showed progressive cortical senescence.

It is not known to what degree tomato is representative of dicotyledonous plants in these respects. Tomato roots are easily cultured *in vitro* (Street, 1957), whereas roots of several other

plant species cannot be maintained continuously in equivalent culture conditions. Nevertheless, it remains true that no progressive programmed root cortical death equivalent to that in cereals and grasses has been unequivocally demonstrated for dicotyledonous plants.

In further experiments in this section the nature of RCD and possible ways to manipulate it were studied, attention being focused on wheat roots. Perhaps the most important general finding from these studies was that the rate of RCD could be affected by many factors but its characteristic pattern was not markedly affected by any treatment. Using intact plants on mineral agar, removal of the seed had no overall significant effect on the rate of RCD, although there was an interaction between the effect of seed removal and sampling time, which cannot easily be explained (Table 3.5). Removal of the shoot or the root meristem had a much greater effect overall (although still amounting to only a fraction of one cell layer) and the interesting feature of this was that it caused a <u>decrease</u> in RCD. Possible reasons for this are discussed in Section 4 which reports studies on glasshouse-grown plants. Briefly, the basis of an explanation probably must be sought in indirect rather than direct effects of the treatments. Henry & Deacon (1981) suggested that the root cortex dies because it does not receive a continuing supply of plant assimilates. Barlow (1982) noted that the evidence for this is weak. The connection of senescence with lack of assimilates -i.e. senescence due to starvation- was first proposed by Molisch (1938) to explain the monocarpic senescence of plants but this theory is now debatable (Nooden, 1988a; Nooden *et al.*,1978). McPherson (1939) attributed cortical senescence of maize roots to starvation, but this was rather a process of aerenhyma formation. To test their

hypothesis, Lewis & Deacon (1982) shaded the shoots of wheat and barley seedlings; contrary to expectations this was found to delay RCD. The proposed explanation was that shading markedly reduced the rate of new root production so that, despite the reduction of the total amount of assimilates reaching the root system, the existing roots (which were assessed for RCD) received proportionally more assimilate from shaded than from unshaded plants, resulting in slower rate of RCD. Similar results were obtained from wheat plants growing in a glasshouse when the shoots were severely pruned (Section 4.4). The effects of excision of shoots, reported here, could be explained on the same basis if it is assumed that the presence of a shoot promotes root growth to the detriment of the cortex of existing root regions. The effect of excision of root meristems in reducing RCD could be explained if the meristem acts as a nutrient sink, either restricting the continued allocation of nutrients to the existing root cortex or actually drawing nutrients from the existing cortex, hastening the senescence. There is no direct evidence for remobilisation of nutrients from the senescing cortex to other root tissues, but this might be investigated in future work by using radioactive tracers.

The results of the experiment where root pieces were incubated on split-plates with different media showed that the presence of sugar at the apical part of the root piece, when the root tip was attached, decreased the amount of RCD in areas remote from the tip. On the other hand, in root pieces with tips removed, the medium at the base significantly decreased RCD in areas remote from the base (see analysis in Appendix 8). These results indicate nutrient mobilisation along the root. We cannot, however, say if this mobilisation occurs from the phloem or the cortex, via a symplastic or apoplastic route.

Attempts to manipulate the rate of RCD in detached roots involved the addition of sucrose, minerals supplied from the basal or apical part of the root piece, indolylacetic acid, gibberellic acid (GA_3), benzylaminopurine, $AgNO_3$, $CoCl_2$, sodium benzoate, gallic acid, ascorbic acid and cycloheximide to agar on which the root pieces were supported.

Even 2% sucrose when present alone or with other factors and supplied along the whole length of root pieces had only a relatively small effect in delaying RCD of wheat, whereas a much lower concentration of sucrose fully maintained the viability of tomato roots. There is a possibility that cortical cells of wheat are unable to use sucrose applied externally, as Dick & ap Rees (1975) reported for pea roots. But various sources of sugar must have been tested in attempts to maintain cereal roots in permanent "root organ culture" which is still not possible. A more likely explanation is that RCD in cereals is genetically programmed and can be influenced only partly by external factors.

Aging and senescence in higher plants are genetically as well as environmentally regulated processes, intimately associated with hormonal interactions. There is a voluminous literature on relationships between plant growth regulators with senescence, reviewed by Mattoo & Aharoni, (1988); Van Staden *et al.*, (1988) and Nooden, (1988b).

Of all substances tested here, IAA had the most marked effect in delaying RCD, especially in the older parts of root pieces (Table 3.9) and at a concentration of 10^{-5} M; the reduction of RCD in this case amounted to more than one cortical cell layer. Auxins are not considered as being primary involved in senescence processes as are cytokinins and ethylene, but they can either retard senescence, or

promote it during xylem differentiation or in floral tissues (see Nooden, 1988b for review).

Cytokinins play an important role in the senescence of different plant organs (Van Staden *et al.*, 1988). The fact that they are produced mainly in roots and that the root apex contains substantial amounts of cytokinins makes the study of cytokinins particularly interesting in relation to senescence of the root cortex. Several reports (cited in Van Staden *et al.*, 1988) indicate that natural or synthetic cytokinins are not readily transported from sites of application unless they enter the transpiration stream. So once the xylem elements differentiate a few millimetres behind the root tip, cytokinins produced by the tip may be transported via the xylem and possibly not reach the outer, differentiated root cortex, leading to RCD. However, the removal of root tips in the experiments with root pieces in this section was found to reduce the rate of RCD. Also, application of benzylaminopurine caused an increase in rate of RCD in root pieces (Table 3.9). The implications of these findings is that root-tip produced cytokinins may hasten RCD, although there are interpretational problems relating both to an interaction between cytokinins and sucrose (Table 3.10) and the effects of cytokinins on growth of root tips and of laterals. Gillespie (1986) observed a decrease in rate of RCD and a loss of the polarity of RCD along root pieces (RCD was uniform along them) when kinetin was supplied in the supporting agar but this could have been due to an effect of the cytokinin in suppressing the development of root laterals (potential sinks for nutrients remobilised from existing regions of root pieces). Benzylaminopurine did not change the polarity of RCD in my work, perhaps because it acts differently from kinetin or the

experimental conditions were different. The effect of BAP on RCD in my work seemed to be "direct" rather than indirect because the root pieces did not produce many laterals on sugar-free medium and, in any case, RCD was enhanced by BAP.

Gibberellins can also affect senescence of plant parts but there is a high diversity of responses to gibberellins in senescence processes in different plant species and different systems (Nooden, 1988b). The findings from the experiment in Section 3.7 show that gibberellins promote RCD in wheat root pieces.

The role of ethylene on senescence in plants is well established (Lieberman, 1979; Mattoo & Aharoni, 1988), especially with regard to fruit ripening and leaf senescence. Ethylene mediates aerenhyma formation in the maize root cortex (Jackson *et al.*, 1985), a process that involves a certain pattern of senescence and death of cortical cells that is, however, different to that in RCD (Deacon *et al.*, 1986). Production of ethylene is a common response of plants to several types of environmental stress. Excision of plant organs in many cases causes increase in the rate of ethylene production (Matoo & Aharoni, 1988). Ethylene-mediated RCD might thus explain the increased RCD of root pieces as opposed to attached roots observed in experiments in this section, or the increased RCD in impeded roots (Kirk & Deacon, 1986).

Cobalt ion is an inhibitor of ethylene biosynthesis (Yu & Yang, 1979), whereas Ag^{+} has a dual effect in stimulating ethylene biosynthesis and inhibiting senescence by antagonising ethylene action (Matoo & Lieberman, 1982). When either cobalt or silver ions were sprayed or infiltrated at non-toxic concentrations into fruit prior to harvest they delayed but did not prevent fruit ripening (Wang & Mellenthin, 1977). Cobalt ion had the most interesting

effect on RCD in wheat root pieces. At a concentration of 20 μM it significantly reduced RCD, and to a lesser degree at 10 μM. Silver ion also reduced RCD in all concentrations used. These are strong indications that ethylene is connected with RCD in root pieces.

Ultrastructural studies and work on membrane permeability suggest that senescence in plants is related to the loss of integrity of cellular membranes even early in the senescence process (Eze *et al.*, 1986). This could be caused by free radicals, the levels of which increase in senescing plant tissues. Membranes could be expected to be highly prone to damage by free radicals causing peroxidation of unsaturated fatty acids (Kellog & Fridovich, 1975). The effects of free radical scavengers in these systems also suggest that free radicals are involved in senescence (Baker *et al.*, 1977; Wang & Baker, 1979). Ascorbic acid is a water soluble antioxidant which regenerates vitamin E in leaf tissues (Finckh & Kunert, 1985). One of the roles of this membrane-associated vitamin is to scavenge free radicals (Tappel, 1962). However, ascorbic acid had no effect on RCD in my work, and the free radical scavengers, sodium benzoate and gallic acid, had a toxic effect on cortical cells, especially at concentrations higher than 0.25 mM (Table 3.12). These findings cannot easily be interpreted but they indicate that RCD may be an orderly process of senescence, where the cell membranes retain their integrity for some time and there is possibly even a withdrawal of nutrients from these cells before they lose function.

Cycloheximide inhibits elongation of peptide chains on 80S ribosomes and has been found to inhibit senescence (chlorophyll and protein loss) in leaf disks (Shibaoka & Thimman, 1970) and ripening in disks of fruit tissue (Frenkel *et al.*, 1968). This was interpreted as an indication that senescence is an orchestrated

process requiring the involvement of macromolecules in a dynamic state and not simply a phenomenon of breakdown. In root pieces cycloheximide had very little effect on RCD so it seems that this process is not dependent on biosynthesis of new macromolecules. If hydrolases are involved in RCD, then these must be preformed and released or activated during the process of RCD. There are, however, various side effects of cycloheximide that make interpretations difficult (Brady, 1988).

The following general conclusions are drawn from all these experiments on the effects of various treatments on RCD.

RCD occurs in both attached and detached roots but detached roots show an increased rate of RCD. The rate can also be altered by various treatments, but only to a limited degree. The general pattern of RCD remains the same, suggesting that RCD is a highly regulated and programmed phenomenon. Moreover, it is not simply explicable in terms of the actions of individual hormones or nutrients, even though many of these can be shown to affect the rate of RCD to some degree. In some respects, these findings were disappointing because it was hoped to manipulate the rate of RCD experimentally and thereby to study the relationship between RCD and invasion of roots by microorganisms in very closely controlled conditions. In the event, most of the work in this thesis had to depend on correlations between RCD and invasion by fungi, because RCD could not be manipulated to a high and consistent degree.

The rate of invasion of wheat root pieces by *M. bolleyi* was always related to the amount of RCD found in control (non-inoculated) root pieces pre-incubated for different times, suggesting that invasion by *M. bolleyi* is largely governed by RCD. Kirk & Deacon (1987b) found also that *M. bolleyi* depends on RCD for

invasion of the root cortex in glasshouse experiments. They also showed that *M. bolleyi* does not enhance cortical senescence in artificially inoculated wheat and grass. From the results of the experiments in this section, however, (Figures 3.19 & 3.33) it is clear that *M. bolleyi* accelerates the death of cortical cells in root pieces, but only by one or two cell layers ahead of that which occurs in natural RCD. The depth of hyphal penetration in the cortex reflects this enhancement of cell death. Since the loss of nuclei assessed with AO probably reflects a late stage, at which cells have lost all contents and regulation, it is possible that the fungus only accelerates the death of cells that were in the course of senescence in any case. In support of this view, the fungus penetrated rapidly into the epidermis and outer cortex of freshly excised root pieces (0 days pre-incubation) placed on fungal colonies. But when the fungus reached the inner cortical layers, there was a substantial delay in further penetration. The same happened but to a lesser degree in root pieces pre-incubated for 2 days, whereas there is no sign of resistance in any cortical cell layer in root pieces pre-incubated for 4 days. In all cases, as shown by Henry & Deacon (1981), the innermost cortical cell layer (next to the endodermis) tended to retain nuclei long after the five outer layers had died. The resistance to fungal invasion shown in this cell layer may reflect its delay to senesce or chemically based unsuitability for *M. bolleyi*.

The characteristic black chlamydospore-like bodies of *M. bolleyi* were produced abundantly on the surface of freshly excised root pieces that hindered the assessment with AO. They were produced to a lesser degree, later and deeper in the cortex, in root pieces pre-incubated for 2 or 4 days. These results are similar to

those reported by Kirk & Deacon (1987b) who found that the dark cells of *M. bolleyi* were formed in senescent cortical cells next to the outermost living cortical cells.

The dependence of *M. bolleyi* on RCD for invasion of the cortex was shown also in the experiment with roots attached to or detached from the seedlings. Roots attached to seedlings showed less RCD than that shown by excised root pieces in the same conditions. In the attached roots, *M. bolleyi* had only a small, non-significant effect on the rate of RCD, even from agar inocula, and fungal hyphae were found only in the outer one or two cell layers.

In a further experiment *M. bolleyi* was found to overcome the resistance of detached roots but not of attached roots of all four small-grained cereals, but again mainly accelerating the senescence that would have occurred in any case. The implication of all these findings is that *M. bolleyi* is a weak parasite rather than an aggressive invader of root tissues.

P. lycopersici showed an interesting difference in behaviour from that of *M. bolleyi*. From spore inocula it did not enhance RCD of cereals and penetrated only the epidermis of both attached and detached roots until the latter began to senesce. From agar inocula, however, *P. lycopersici* caused much more damage than *M. bolleyi* in both attached and detached roots. Then it significantly increased RCD from the second day of incubation and the degree of hyphal penetration was also higher than by *M. bolleyi*. These results were unexpected because *P. lycopersici* does not naturally occur on cereals. The results were probably caused by an overwhelming inoculum level but they nevertheless demonstrate a higher invasive (pathogenic) potential by *P. lycopersici* than by *M. bolleyi*.

Tomato roots were included in these experiments in an attempt to detect differences in resistance to attack by the two fungi which could be attributed to senescence. Control tomato roots, attached or detached from seedlings, lost viability only of the epidermal cells during 7 days' incubation. Inoculation with *P. lycopersici* (agar inocula), however, led to rapid invasion and death of root cells in 24 hours, in both attached and detached roots. The attached and detached roots showed some difference in resistance when inoculated with spores of *P. lycopersici*. The fungus invaded and killed all roots eventually but it killed the cortex of detached roots up to the innermost cortical cell layer in 4 days, whereas 7 days were required for similar damage in the attached roots. This reflects a difference in resistance to invasion in roots of different physiological condition which can be called senescence (due to excision) in a general concept of the term. A difference in aggressiveness of *M. bolleyi* and *P. lycopersici* was again seen in tomato roots. Roots attached to seedlings were largely unaffected by *M. bolleyi* and retained their cortical viability whereas *M. bolleyi* caused cell death of detached tomato roots 4 - 5 days after inoculation. These results, especially from spore inocula, show clearly that *M. bolleyi* can invade and damage root tissues that are weakened by stress conditions but not those that retain full vigour. An interesting finding in this experiment, which has yet to be explained, is that tomato roots killed by *M. bolleyi* were not penetrated by fungal hyphae beyond the epidermis.

An attempt was made to compare the resistance of wheat, barley, rye and oat roots to invasion by *M. bolleyi* and *Gaeumannomyces graminis* var *tritici*. Of interest in this experiment was the difference in RCD shown even by uninoculated roots of the cereals,

which are reported to show characteristic differences in rate of RCD in normal soil conditions (Deacon & Mitchell, 1985; Yeates & Parker, 1986). Although there were some differences in the pattern of RCD between the cereals and in the mean number of cortical cell layers, which complicated interpretations, rye showed a much lesser rate of RCD than did wheat and barley in both attached and detached roots. Also, barley showed a somewhat lower rate of RCD than did wheat in both attached and detached roots. Oats, however, showed unusual behaviour, in that the epidermal cells retained viability for almost as long as did the other cortical cell layers; as RCD progressed, so the nuclear status of the inner cortical cells declined at much the same rate as that of the epidermis. This pattern clearly differs from that in the other cereals and in non-cereal grasses that have been examined to date. But Smiley & Giblin (1986) noted a similar pattern of senescence to this in roots of *Poa pratensis* when the plants were subjected to high temperatures above the optimum for plant growth. The fact that the "normal" centripetal progression of RCD described for other cereals was not found in even attached roots of oat suggests a need to re-evaluate the universality of this normal pattern. It may be said, however, that the conditions of these experiments were unusual; the seedlings were in darkness. Smiley & Giblin (1986) noted that shading of *Poa pratensis* turf was associated with senescence of the root cortex and invasion by a fungus (stated to be *Phialophora graminicola* but now known to be *Magnaporthe poae*, Landschoot & Jackson (1989)). Perhaps the pattern of RCD is influenced by unusual environmental conditions. In this respect, also, it is notable that the development of aerenchyma in maize roots is associated with an "unusual" pattern of nuclear deletion from cortical cells, which tends to start in the middle

cortex rather than in the root epidermis. Deacon *et al.* (1986), found that maize roots undergoing aerenchyma formation could show both a "normal" pattern of RCD (beginning in the epidermis) and an "unusual" pattern associated with aerenchyma formation and starting in the mid cortex.

Inoculation of attached roots of the four cereals with *M. bolleyi* did not lead to a marked increase in RCD although the fungus did accelerate the rate of death in the outer cortex. In contrast, *M. bolleyi* caused a rapid loss of cortical viability in detached roots of all cereals - even more marked in barley, rye and oats than in wheat, despite the fact that uninoculated detached roots of rye, for example, tended to retain nuclei longer that did wheat. These findings might be taken to indicate that differences in persistence of nuclei in detached roots of the cereals are not necessarily related to major differences in resistance of the cells of the respective cereals to resist invasion by parasites. But this interpretation is not borne out by observations on the depth of penetration by *M. bolleyi*.

The oat root cortex seems to resist invasion by hyphae of *M. bolleyi*. Even 10 days after inoculation, the fungus had not penetrated further than the epidermis in either attached or detached roots, while wheat, barley and rye roots attached to seedlings were penetrated especially after the sixth day by intercellular hyphae, and in detached roots this happened earlier.

There was a clear difference in damage to the cereals by *G. graminis* var. tritici. Wheat and barley were most damaged, oats and rye least damaged. Also, oat was more resistant than rye to *Ggt* For example, the resistance of rye could be overcome by the higher

inoculum level provided by PDA than by WA inocula whereas this was not true for oat.

In the case of rye, both detached and attached roots showed early resistance to *Ggt* (from WA inocula) but this resistance was lost by 4 days. In the experiment as a whole it was found that detached roots could be used to differentiate the resistance of cereals to *Ggt*, but only in the first 2 days of incubation, the effects being lost at 4 days except in the case of oats.

The resistance of oats to *Ggt* was remarkable, compared with the resistance of the other cereals to this pathogen and even when compared with the relative susceptibility of detached oat roots to *M. bolleyi* (as evidenced by rapid loss of root cell viability but not by invasion by *M. bolleyi*. This resistance of oats is usually ascribed to saponins such as avenacin (Turner, 1956; Holden, 1980) which are known to inhibit *Ggt* but have lesser effects on *M. bolleyi* (Section 6.6.3). The resistance mechanisms that restrict *M. bolleyi* to the outer cortex of the cereal roots until cortical senescence advances and which slow penetration the unrelated pathogen *P. lycopersici* and the pathogen *Ggt*. remain unknown. Lignification reactions may be involved, and they were seen in all experiments here with root inoculations. Ride & Pierce (1979) showed that one of the major effects of lignification may be to prevent the actions of parasite-derived enzymes such as cellulase on the plant cell walls because lignin-like polymers physically shield the wall polymers from enzyme or chemical attack.

Intense lignification reactions were detected with phloroglucinol-HCl in roots attached to seedlings and challenged with heavy inocula of *M. bolleyi* or *P. lycopersici*, whereas less or no lignification occurred when roots were inoculated with spores.

Less lignification occurred in cortices of root pieces than in roots attached to seedlings, suggesting that this defence mechanism depends on cortical cell viability and perhaps access to nutrient resources.

From all the above, it seems that a fungus, in order to cause intense lignification in cortical cells, must be present at a sufficiently high inoculum level and before the root cortex senesces. On the other hand, the fungus that elicits lignification reactions is potentially disadvantaged because lignified cell walls are the more resistant to enzymatic attack. There has been much debate as to whether localised lignification reactions such as those seen in papillae are an effective means of host defence (Skou, 1981). The lignitubers formed in response to invasion by *P. lycopersici* were readily penetrated (Fig. 3.22) whereas those formed in response to *M. bolleyi* seemed to restrict invasion. But *M. bolleyi* was seen often to penetrate the cortex intercellularly, at least in the early stages of invasion of root pieces. It may be postulated that this intercellular mode of growth by *M. bolleyi* is an adaptation to (or a consequence of) the need to circumvent host resistance reactions. Deacon (1980) argued that the characteristic ectotrophic infection habit of *P. graminicola* is such an adaptation of a fungus that exploits naturally senescing cortical tissues of cereals and grasses, such that the fungus always grows next to the outermost layer of living cortical cells and can invade them readily as their resistance declines. In this respect Tables 3.29 and 3.30 show that longitudinally orientated intercellular hyphae of *M. bolleyi* were always found deeper in the cortex than were intracellular hyphae in both attached and detached cereal roots as their cortices senesced with prolonged time of incubation (6 and 8

days for attached roots, 4 and 6 days for detached roots). The interpretation of this behaviour in detached roots is complicated by the fact that intercellular hyphae could have gained entry from the severed ends of the roots, but still the same phenomenon was seen to a lesser degree in attached roots, where this artificial means of invasion would not have occurred.

A final point of note is that autofluorescence of cortical cell walls, indicative of lignification or other phenolic-based reactions (Holland & Fulcher, 1971) occurred in both uninoculated and inoculated cereal roots. In many instances these reactions were enhanced by inoculation. They were then seen to occur earlier, to be more intense and to occur more uniformly across the cortex than in uninoculated roots. But it was often observed that they occurred only in living cortical cells at depths to which fungal hyphae had invaded, not far in advance of penetration of the cortex by the fungi. The intense stelar lignification that Speakman & Lewis (1978) recorded in wheat roots inoculated with *P. graminicola* may be different in this respect because the presence of the fungus in the outer cortex was found to elicit intense stelar lignification. But in the present work both lignification (assessed by phloroghicinol-HCl) and yellow autofluorescence of the cortex were largely localised phenomena dependent on the close physical presence of a fungus. The role that such reactions play in biocontrol by *P. graminicola* and *M. bolleyi* has not been satisfactorily established. Kirk & Deacon (1987a) have argued that biocontrol by these fungi may operate, at least in part, by competition for nutrients derived from senescing root cortical cells. It seems likely, however, that both competition and lignification reactions could contribute to biocontrol by these weak parasites.

4. Root cortical senescence in glasshouse-grown plants and its relationship with root infection

4.1. Introduction

Early root cortical death (RCD) seems to occur universally in graminaceous plants. As noted in the Introduction and in Section 3, RCD follows a fixed pattern in cereals and grasses, but the factors that govern this pattern and the effects of environmental factors on the pattern and rate of cortical senescence have not been adequately studied. It was first suggested that RCD occurs due to depletion of assimilates in cortical cells (Henry & Deacon, 1981). Treatments such as shading or powdery mildew infection, however, did not enhance the rate of RCD (Lewis & Deacon, 1982; Deacon & Mitchell, 1985).

Mineral nutrient availability is reported to have marked effects on the progress of infection of cereal roots by fungi, most notably by the take-all fungus and by vesicular arbuscular mycorrhizal fungi (Huber, 1981, Mosse *et al.*, 1981). Gillespie (1986) studied some aspects of mineral nutrition and of hydrogen ion concentration on RCD in glasshouse - grown plants, in an attempt to relate such effects to infection. He found that a reduced supply or omission of N (as NO_3^- or NH_4^+) enhanced the rate of RCD, whereas P and K had lesser effects. Brown & Hornby (1987) also reported differing effects of ammonium- and nitrate-nitrogen on the rate of RCD in wheat in gnotobiotic conditions. MacLeod *et al.* (1986) reported that RCD in wheat was influenced by phosphorus supply to soil, but interpretation of the results was complicated by possible effects of phosphorus on the pattern of plant growth. There are also reports of influences of temperature on RCD (Yeates & Parker, 1986) and that supraoptimal temperatures cause rapid and atypical patterns of death in grass roots (Smiley & Giblin, 1986).

The experiments in this part of the thesis were designed to investigate the effects of mineral nutrients in the root environment or of urea supplied through the foliage and also the effects of reduced supply of photosynthates caused by reduction of the photosynthetic area on the progress of RCD in wheat. The experiments complement those in Section 3 which were based on study of RCD in root pieces and young seedlings in gnotobiotic conditions. The aim of this research was to find possible ways of manipulating RCD for studies on colonisation of senescing roots by pathogenic and non-pathogenic root-infecting fungi, and so to aid interpretation of some often-reported effects of environment on fungal infection roots.

For mineral nutrients, the effects of reduced NO_3-N and Ca nutrition were studied, because N has been reported to affect RCD in wheat (Gillespie, 1986) and Ca is generally involved in senescence processes in plants (Poovaiah, 1988; Leshem, 1987; Ferguson, 1984.

Finally, in this section are included experiments with tomato plants involving inoculation of plants at different ages with spores of *P. lycopersici*, removal of the shoot, and study of death of the cortex. These experiments were done to complement the studies on detached root pieces in Section 3, and they represent some of the few studies aimed at investigating the relationship between cortical senescense and invasion of the roots of dicotyledonous plants by root-infecting fungi.

4.2. Experiment 1: effects of nitrogen and calcium level on cortical senescence in wheat roots

Perspex tubes of 4.5 cm diameter and 30 cm long, filled with horticultural perlite and covered with black polyethylene to exclude

light from the roots, were each sown at the top with two surface-sterilised pre-germinated wheat seeds. The perlite was brought to saturation with Hewitt's mineral solution modified for each individual treatment as explained below. The tubes were in a glasshouse at a mean temperature of 20±1 °C. Natural light was supplemented with fluorescent and mercury vapour lights supplying about 6 klux, measured at plant height, in a 16h light : 8h dark cycle. Each tube received 200 ml of mineral nutrient solution every second day.

In modifying the standard mineral solution for changes in NO_3^- and Ca^{++} concentrations it was necessary to maintain constant levels of other minerals. The NO_3^- ion was substituted with Cl^-, and Ca^{++} with Mg^{++}, but because the increased levels of these two ions might affect RCD, further treatments were included in which $MgSO_4$ and $CaCl_2$ were added to the standard mineral solution. Compositions of the mineral nutrient solutions for the five treatments of the experiment are shown in Table 4.1.

Additionally, all solutions contained micronutrients (mM concentration) as follows: $MnSO_4$, 0.01; Fe-EDTA, 0.1; $ZnSO_4$ 0.001; $CuSO_4$, 0.001; H_3BO_3, 0.05; Na_2MoO_4, 0.0005; NaCl, 0.1; $CoCl_2$, 0.0002.

The experiment comprised 30 tubes, 6 for each of five treatments as follows.

(1) Control: mineral nutrient solution as shown in the first column of Table 4.1, termed "standard".

(2) Nitrogen reduced to one sixth, termed "low N" (second column of Table 4.1).

(3) Ca^{++} omitted, termed "No Ca" (3rd column of Table 4.1).

(4) High Mg^{++} (4th column of Table 4.1).

(5) High Cl^- (5th column of Table 4.1).

The pH of all media was adjusted to 5.8 with HCl or NaOH. The wheat plants were sampled when 38 days old and assessed for RCD. For this, the roots were washed under running tap water, perlite particles were carefully removed and the roots were stored in 70% methylated spirits. One plant from each tube was taken at random and its three first-formed seminal roots were assessed along their length after removal of the laterals and staining with acridine orange (Section 2.5). Whole roots were scanned by microscope fields (x100 magnification) each 1.8 mm diam. and the presence or absence of nuclei was recorded for each of the six cortical cell layers outside of the endodermis along the whole root length.

Table 4.1. Concentrations (mM) of the major nutrient elements in nutrient solutions used in Experiment 1.

Mineral	Treatments				
nutrients	Standard	Low N	No Ca	High Mg	High Cl
KNO_3	4	2	4	4	4
$Ca(NO_3)_2$	4	-	-	4	4
$MgSO_4$	1.5	1.5	1.5	1.5	1.5
NaH_2PO_4	1.33	1.33	1.33	1.33	1.33
K_2SO_4	-	1	-	-	-
$CaCl_2$	-	4	-	-	4
$Mg(NO_3)_2$	-	-	4	4	-

4.2.1. Results

At sampling, plants in different treatments showed major differences in shoot growth (Table 4.2), with significantly lower shoot weight and numbers of tillers in the "low N" and "No Ca" than in the control (standard) treatment. Differences in root growth between treatments (except for plants growing in the absence of Ca) were much less obvious and were not recorded in detail. The length of the axes of seminal roots of plants receiving "low N" did not

Table 4.2. Growth of shoot and root systems of wheat plants grown for 28 days in tubes of horticultural perlite and receiving Hewitt's mineral salts solution with different levels of N, Cl and Mg.*

Treatment	Roots		Shoots	
	Length of seminal axes	Number of laterals per root axis	Fresh weight (g)	Number of tillers
Standard	24.6	45.0	2.69	1.9
Low N	23.8	45.1	1.33	0.3
No Ca	11.0	0.0	0.11	0.0
Plus Mg	23.8	44.3	2.72	1.7
Plus Cl	26.3	40.3	2.89	1.9
SED**	0.71	2.71	0.11	0.31
LSD 5%	1.49	5.69	0.24	0.65

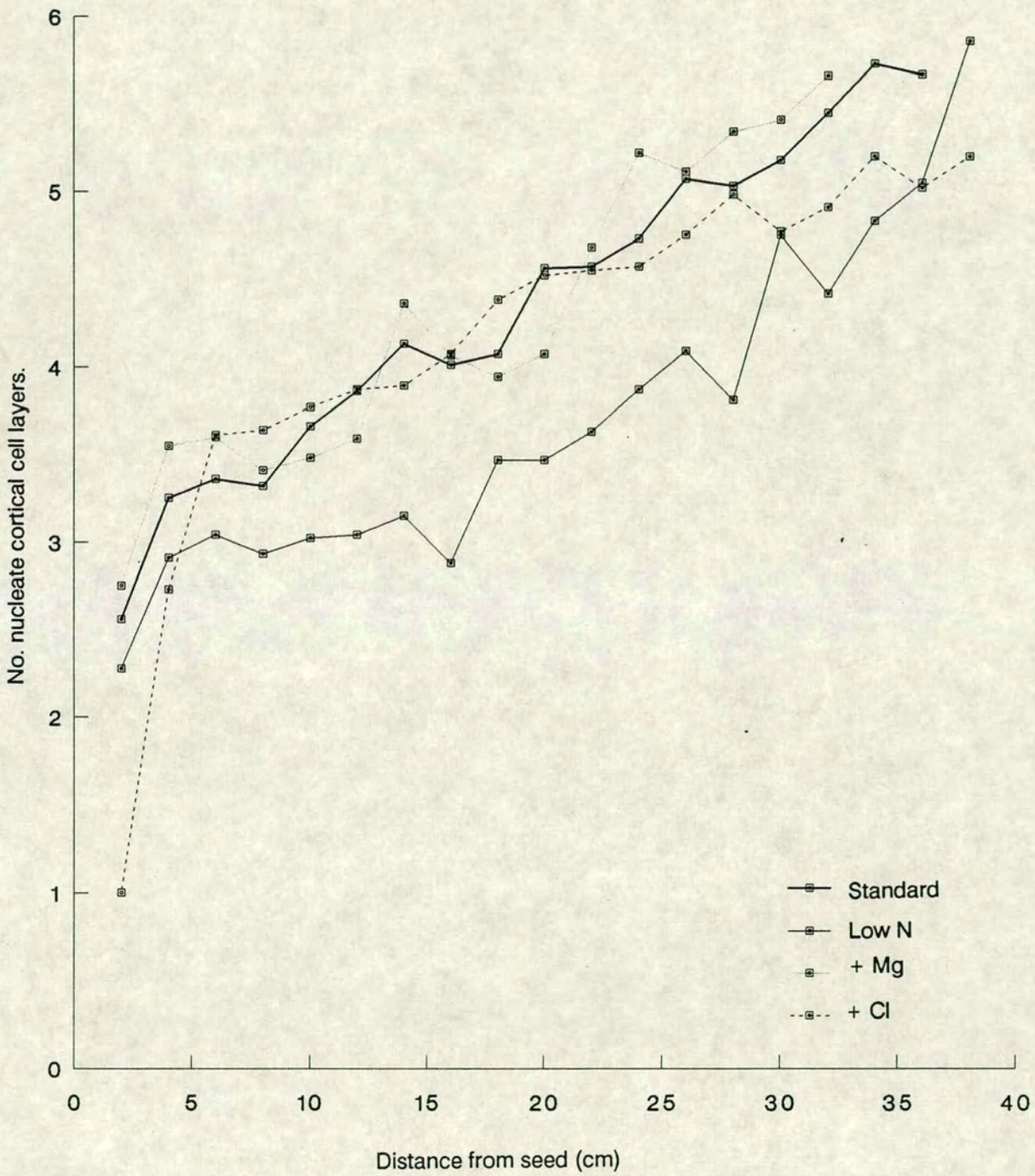
* Means of six replicate plants, three first-formed seminal roots per plant.

** Standard error of the difference between any two means.

differ significantly from that in other treatments, and these roots bore the same numbers of laterals but of poorer total growth. The plants receiving "No Ca" had first very poor growth, and produced very short roots with no laterals. At sampling most of the shoot was chlorotic and the roots were short and brown without laterals. When these roots were assessed for RCD, the cortex was found to be totally dead and only occasionally did some apical regions have cells with stainable nuclei. Plants from this treatment were excluded from further assessments and statistical analysis.

Assessments of RCD are shown in Figure 4.1 as the mean numbers of cortical cell layers (max. 6) that were nucleate along the root length. RCD followed the usual pattern in all treatments. Plants in the "low N" treatment (2 mEq as opposed to 12 mEq of N) showed more RCD than the control plants whereas plants receiving high Mg or high Cl were similar to controls. Excluding the small region closest to the seed (the first data point in Fig.4.1), the amount of RCD was essentially linearly related to distance along the roots in all treatments. The means of the regression coefficients (slopes) from each individual root in this experiment (3 roots from each of the 6 replicate plants in each treatment) were as follows: 0.21, 0.17, 0.16 and 0.24 for standard, low N, high Cl and high Mg treatments respectively. They did not differ significantly from one another by analysis of variance. But the mean of the regression constant (*y intercept*) in the "low N" treatment, (4.5) was significantly lower (P=0.005) than that in the other three treatments (5.5, 5.5, and 5.7). This shows that the pattern of RCD did not change, but the whole process was accelerated in conditions of reduced supply of nitrogen.

Figure 4.1. Number of nucleate cortical cell layers along seminal root axes of wheat seedlings grown for 38 days in perlite containing complete mineral salt solution (standard), or solution with reduced N content, or increased Mg^{2+} or Cl^- content. Each point represents the mean of 6 replicate plants, 3 roots per plant.



As the plants grown with increased levels of $MgNO_3$ or $CaCl_2$ did not show any difference in RCD from plants receiving the standard solution, these two salts were used instead of $Ca(NO_3)_2$ in subsequent experiments when it was necessary to manipulate nitrogen or calcium levels.

4.3. Experiment 2: calcium nutrition and inoculation of wheat roots with *M. bolleyi*

The two primary aims of this experiment were to compare the effects of different levels of Ca^{++} on the rate of RCD in wheat and to assess its implications for susceptibility of the root cortex to invasion by *M. bolleyi*.

Because of the very poor growth of the plants in nutrient solution without calcium in the previous experiment, the effects of reduced calcium levels were tested in this experiment. The standard solution was as used previously (8 mEq Ca^{++}) but variations were as follows: twofold calcium (16 mEq Ca^{++}), one-third calcium (2.7 mEq Ca^{++}), one-ninth calcium (0.9 mEq Ca^{++}), and one twenty-seventh calcium (0.3 mEq Ca^{++}). For these purposes $Mg(NO_3)_2$ was substituted for $Ca(NO_3)_2$ to maintain the nitrogen level, and the surplus calcium in the twofold calcium treatment was supplied as $CaCl_2$. Table 4.3 shows the compositions of the major elements in the different treatments.

Based on the results of the previous experiment, this experiment was done in 40 cm lengths of perspex tubing in order to ensure a larger rooting depth. There were 6 replicate tubes per treatment, two plants per tube. At sampling, the three first-formed seminal root axes from one plant in each tube were assessed for RCD.

Part of this experiment was also designed to study the effect of enhanced RCD on the extent of root colonisation by *M.bolleyi*, and the effect, if any, of supplying a carbon source to the fungus on its ability to colonise roots.

Table 4.3. Concentration of the major elements in nutrient solutions used in Experiment 2. (Mm)

Mineral nutrients	Treatments				
	2 Ca	Standard	1/3 Ca	1/9 Ca	1/27 Ca
KNO_3	4	4	4	4	4
$Ca(NO_3)_2$	4	4	1.3	0.44	0.15
$MgSO_4$	1.5	1.5	1.5	1.5	1.5
NaH_2PO_4	1.33	1.33	1.33	1.33	1.33
$CaCl_2$	4	-	-	-	-
$Mg(NO_3)_2$	-	-	2.66	3.56	3.85

The fungus (strain T560R1) was grown in 1% molasses in shaken liquid culture for 6 days then the culture was filtered through a double layer of muslin and the filtrate was centrifuged at 5000 rpm for 15 min. The spore pellet was resuspended in distilled water, centrifuged again and resuspended in distilled water. The washed spores were added to mineral nutrient solutions of different calcium content (as described earlier) to give a final concentration of 10^4 spores per ml. These suspensions, with or without a supplement of glucose (1% final concentration), were used to inoculate 12 day old wheat plants by adding 400 ml of spore suspension to the top of each tube so that it percolated down. There were two inoculated tubes per

calcium treatment, one with and one without a glucose supplement. Before and after the initial inoculation, all tubes received 200 ml mineral nutrient solution (without glucose) at 2 day intervals.

A treatment of shading was also included. Five wheat plants, one from each Ca-treatment, were shaded by means of green plastic netting placed around the plants and between them and the lights, so reducing the illumination to one third of control values. This part of the experiment was treated as independent from the rest of the experiment. It was designed and analysed as a 4X4 factorial, namely 4 levels of Ca^{2+} and four treatments (none, shading, inoculation and inoculation with glucose).

When the plants were 30d old, they were sampled and the roots assessed for RCD as described earlier. They were also assessed for colonisation by *M. bolleyi* by recording the number of groups of brown chlamydospore-like bodies for each microscope field along the root length. The first three seminal roots of each plant were assessed.

4.3.1. Results

Tables 4.4 and 4.6 show the effects of calcium content of mineral nutrient solutions on the growth of plants. Plants grown with the lowest concentration of calcium had very poor growth much like the plants grown in the absence of calcium in the previous experiment. Their roots were brown and the cortical cells anucleate. Results from plants of this treatment were not included in further comparisons and statistical analysis. The reduction of Ca to 1/9 of the standard concentration very significantly reduced the growth of shoots and the lengths of seminal root axes. The reduction of Ca to 1/3 of standard concentration affected the shoot growth to a lesser

Table 4.4. Growth of shoot and root systems of wheat plants after 30 days in tubes of horticultural perlite receiving Hewitt's mineral salts solution with different levels of calcium.*

Treatment	Length of root axes (cm)	Shoots	
		Fresh weight (g)	Number of tillers
2 X Ca	43.8	2.55	2.1
Standard	42.6	2.42	2.0
1/3 Ca	37.4	1.20	0.9
1/9 Ca	21.3	0.25	0.2
1/27 Ca	N D	0.14	0.0
SED**	2.17	0.11	0.17
LSD 5%	4.34	0.23	0.36

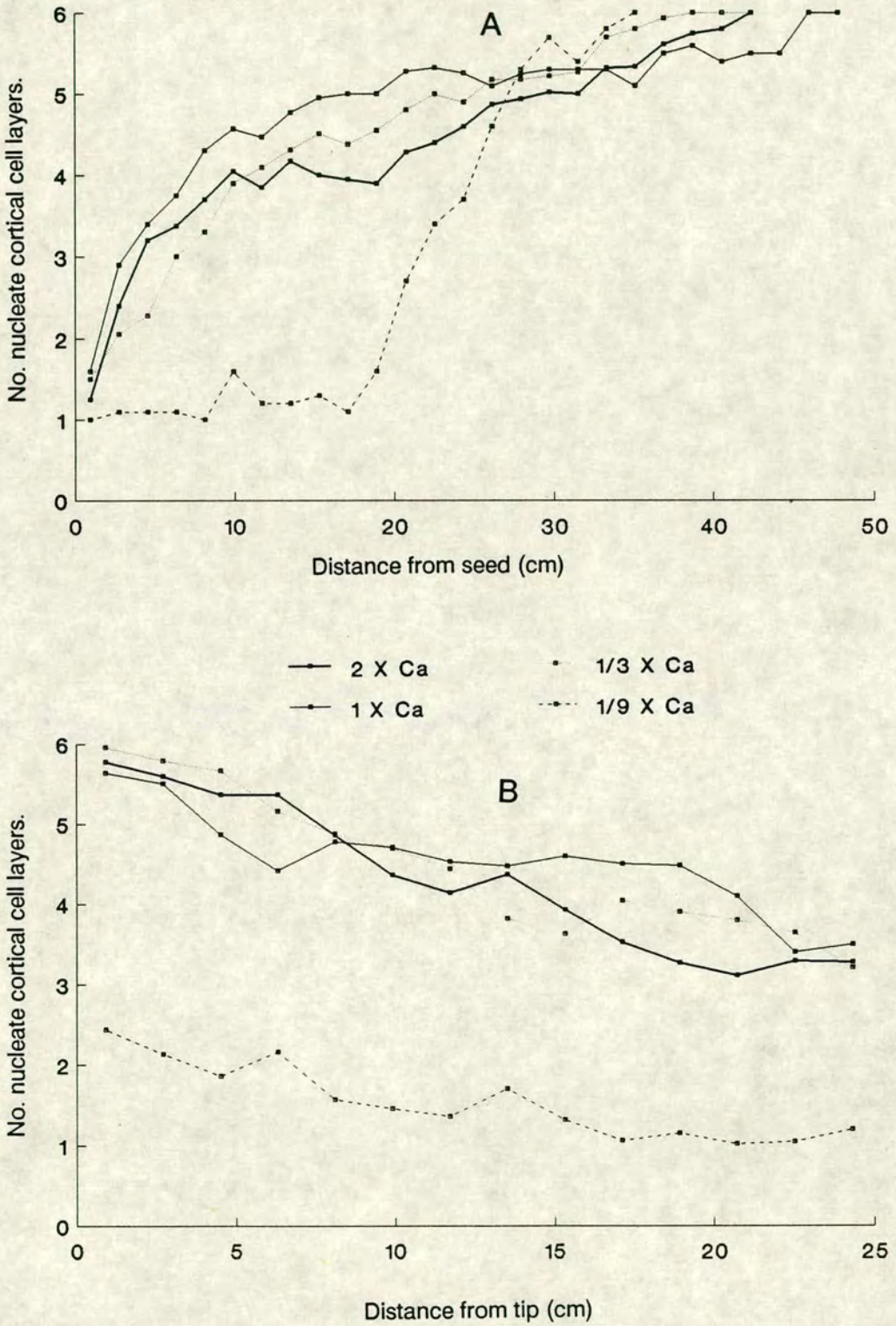
* Means of six replicate plants.

** Standard error of the difference between any two means.

degree and it did not significantly affect the root length. The treatment involving twofold Ca concentration did not significantly affect the growth of the plants.

Figure 4.2 shows the amounts of RCD in plants supplied with different amounts of Ca. The data refer to the three first-formed seminal roots (central and first pair) in each of six replicate plants. When the concentration of calcium was doubled or reduced to 1/3 of standard it did not affect significantly the amount of RCD, but in the treatment with 1/9 Ca there was a dramatic increase of RCD. To analyse this latter effect, account had to be taken of the effect of low calcium level on the rate of root growth. So the results for RCD were considered in two ways. First the amount of RCD was compared between different treatments for parts of the roots at the same distance from the seed (Fig. 4.2 A). This is probably most valid for comparisons of the older parts of the roots where cumulative effects of different rates of root growth would be least. Second, to enable comparisons of the younger parts of roots, the amount of RCD was plotted as a function of distance from the root tip (Fig. 4.2B). From the plots it is clear that the 1/9 Ca treatment had a very marked effect in increasing RCD in the younger parts of the roots as well as in their older regions. Taking into account the lower final growth of roots in treatments with reduced Ca, and the fact that roots from the standard and twofold Ca treatments had reached the bottom of the tubes (which accelerates RCD), the differences between the 1/9 Ca and other treatments were probably greater than shown in Fig. 4.2B. The data from this experiment were subjected to statistical analysis as described previously (Section 4.2). For the data of Fig. 4.2A & B, the constants of the linear regression lines of RCD along the root

Figure 4.2. Number of nucleate cortical cell layers in seminal root axes of wheat grown for 30 days in perlite containing mineral salt solution with different levels of Ca^{2+}. Each point represents the mean of 6 replicate plants, 3 roots per plant. A, assessments based on distance from base of root (seed); B, assessments based on distance from tip of root.



length in the 1/9 Ca treatment were statistically different from those of all other treatments ($P<0.001$), while for the slopes there was no significant difference (Table 4.5).

Table 4.5 Regression slopes and *y* intercepts for data in Figures 4.2A & B.

Treatment	Data from Fig. 4.2A		Data from Fig. 4.2B	
	y intercept	Slope	*y* intercept	Slope
2 Ca	2.4	0.17	6.1	-0.22
Standard	2.9	0.18	5.6	-0.13
1/3 Ca	2.0	0.20	6.1	-0.20
1/9 Ca	-0.02	0.26	2.4	-0.10

From the part of the experiment where different Ca levels were combined with shading and inoculation, the Ca-treatments had the same effects on shoot growth as previously (Table 4.6). Two-way analysis of variance showed a highly significant effect of Ca-treatments on shoot fresh weight ($P=0.001$). The mean length of the first-formed seminal roots was also reduced in the 1/9 Ca treatment but the statistical analysis failed to show any significance because of the relatively high variance. The inoculation of roots with *M. bolleyi* did not affect shoot or root growth. Shading reduced the shoot growth but this was not significant statistically. There were no statistical interactions between the treatments.

Table 4.7 shows the amounts of RCD, as the mean number of nucleate cortical layers (max. 6), in the combined treatments of different Ca levels, shading and inoculation with spores of

M. bolleyi. As a basis of comparison between treatments it was necessary to analyse the results (two-way analysis of variance) for the younger and the older halves of the axes separately, in order to minimise the effects of variation in root length between the different treatments as explained previously. Inspection of Table 4.7 shows that shading had no significant effect on RCD. The most pronounced effect on RCD was caused by a reduction of Ca to one-ninth of standard, which greatly enhanced RCD. Increased RCD was observed also in the older parts of the roots of inoculated compared to non-inoculated plants. There was no gross difference in the amount of RCD when spores of *M. bolleyi* were supplemented with glucose or

Table 4.6. Mean fresh weight (g) of shoots of plants growing in perlite with mineral nutrient solutions of different Ca^{++} levels in combination with treatments of shading and inoculation with *M. bolleyi*.*

Ca^{++} Conc.	Treatment				
	None	Shading	*M. bolleyi*	*M. bolleyi* plus 1% glucose	Row mean
X 2	2.33	1.52	2.16	1.82	1.95
Standard	1.81	1.50	1.87	2.08	1.82
X 1/3	1.36	1.05	1.55	1.17	1.27
X 1/9	0.40	0.50	0.40	0.44	0.41
Column mean	1.45	1.12	1.49	1.38	

SED** = 0.21, LSD (5%)=0.48

* Means of two plants per tube.

** SED and LSD for comparisons between any two row means or any two column means.

Table 4.7. Numbers of living cortical cell layers in root axes of wheat plants supplied with different levels of calcium and untreated (control), shaded or inoculated with *M. bolleyi* (Mb) in the presence or absence of a 1% glucose supplement.*

Ca Level	Part of root	Control (no additional treatments)	Shading	Mb alone	Mb plus glucose	Row means
X 2	Young	5.0	4.6	4.3	5.2	*4.7*
	Old	3.0	2.2	1.9	1.9	**2.2**
X 1	Young	5.1	4.7	4.7	5.2	*4.9*
(Standard)	Old	3.5	3.1	2.2	1.5	**2.5**
X 1/3	Young	5.2	5.1	4.2	4.5	*4.8*
	Old	2.8	2.9	2.1	1.4	**2.3**
X 1/9	Young	1.9	3.5	1.7	1.2	*2.1*
	Old	1.1	1.2	1.0	1.0	**1.1**
Column	Young	*4.3*	*4.4*	*3.7*	*4.0*	
mean	Old	**2.6**	**2.3**	**1.8**	**1.4**	

* Results are means of two replicate plants, 3 roots per plant, averaged over the youngest or oldest halves of root lengths. For comparisons between any two column or row means of young parts of the roots (italicised numbers), SED =0.29, LSD (5%)=0.65. For comparisons between any two column or row means of old parts of the roots (bold numbers), SED =0.202, LSD (5%)=0.46.

not supplemented, although there was a trend of increased RCD in the spores+glucose treatment compared with the spores alone treatment in the older parts of the roots.

Characteristic groups of chlamydospore-like structures of *M. bolleyi* were more numerous in roots of plants inoculated with a spore suspension amended with 1% glucose than in those inoculated with spores alone (Table 4.8 & Fig. 4.3A) but in both cases the number declined markedly with distance down the roots. The numbers were lowest in roots supplied with the highest Ca level, which also had the lowest amount of RCD. Conversely, they were relatively high along most of the length of roots of plants in the lowest calcium treatment (Fig. 4.3B).

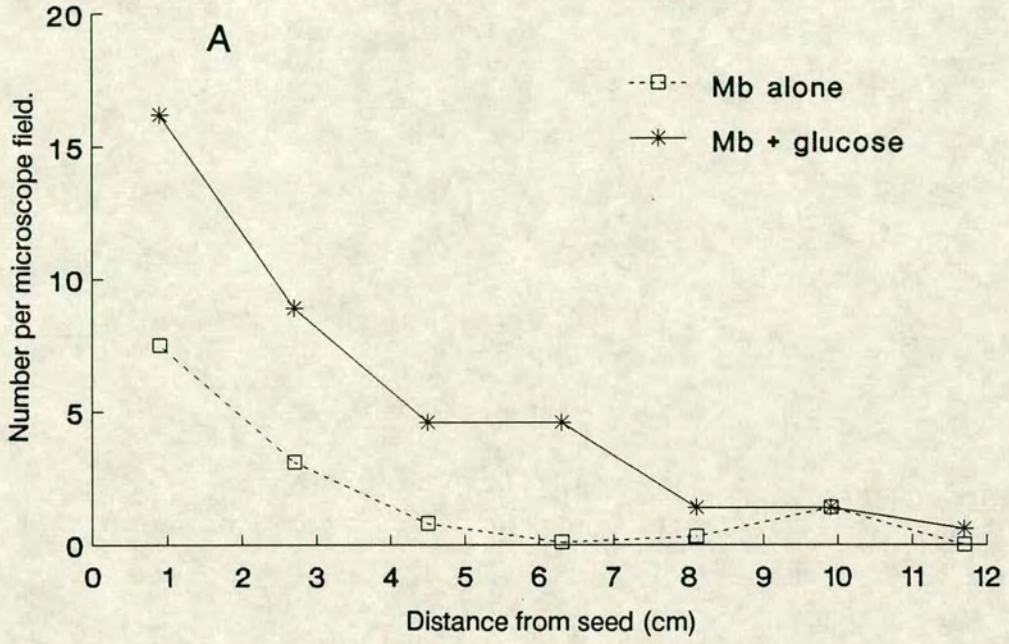
Table 4.8. Numbers of groups of chlamydospore-like structures of *M.bolleyi*, observed in the oldest 10 cm of seminal roots of wheat receiving different levels of Ca and inoculated with spores in the presence or absence of a glucose supplement.*

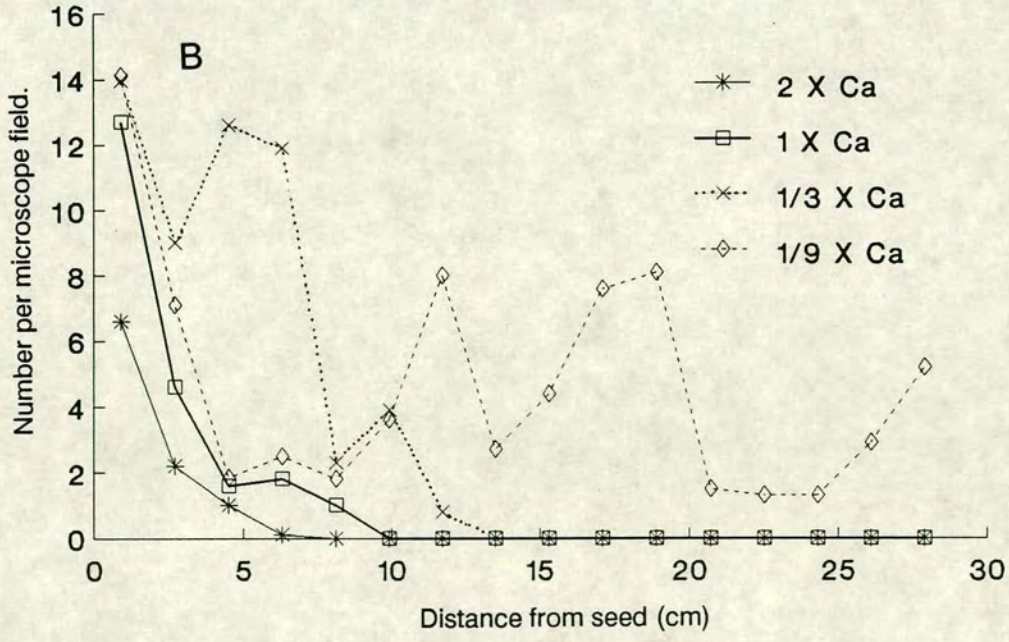
Inoculum treatment	Calcium treatment.			
	X 2	X 1 (Standard)	X 1/3	X 1/9
M.b. no sugar	1.22	1.67	2.09	2.83
M.b. plus sugar	2.35	5.40	11.2	6.57

SED* = 1.15; LSD (5%) = 2.43; LSD (1%) = 3.35

* Means of three seminal roots on each of two plants; SED and LSD for comparisons between any two means.

Figure 4.3. Numbers of groups of chlamydospore-like structures of *M. bolleyi* along axes of wheat seminal roots inoculated with spores of the fungus alone or together with 1% glucose. A, combined data for all calcium supplements, means of 4 replicate tubes, three roots per plant; B, combined data for glucose supplements, means of 2 replicate tubes.





The results from this experiment thus show a major effect of Ca nutrition in maintaining the viability of root cortex. Both the total amount of RCD and its pattern along the root length were affected by a reduction in supply of Ca, and the effect was concentration-dependent. *M. bolleyi* colonised the senescing root cortex and its intensity of colonisation increased as RCD increased (due to Ca depletion). The ability of the fungus to colonise the root cortex was increased when the spore inoculum was supplemented with glucose and inoculation enhanced the amount of RCD in the older parts of the roots.

4.4. Experiment 3: effect of leaf pruning on root cortex death in wheat and on colonisation by *M. bolleyi*

This experiment was designed to study the effect of an acute reduction of the assimilate flow to root systems, caused by a severe reduction of the leaf area, on RCD of wheat seminal axes, and the implications for colonisation of roots by *M. bolleyi*.

Wheat plants were grown, one per tube, in 60 cm lengths of flexible polyethylene tubing filled with horticultural perlite. Natural light was supplemented with fluorescent and mercury-vapour lamps as described in Experiment 1. The tubes received 200 ml of Hewitt's mineral solution every second day.

Treatments were started at the appearance of the first tillers when plants were 26 days old. Then, in a 2X2 factorial design with 8 replicates per treatment, the plants were pruned or not pruned and inoculated or not inoculated with *M. bolleyi*.

For inoculation, the spores from 5 day old liquid cultures in 1% molasses were washed twice in distilled water, suspended in mineral nutrient solution (10^4 spores per ml) and 450 ml of the suspension

was added to the top of each tube so that it percolated down. Strain T560R1 of *M. bolleyi* was again used. Pruning was done by cutting the tops of the plants at 8 cm above soil level, and any subsequent growth above this height was removed every second day, up to the end of the experiment.

An additional eight plants had been grown and were sampled when the treatments started. The roots of this initial sampling were stored in 70% methylated spirits and assessed with the treated plants at the end of the experiment.

When the plants were 43 days old (17 days after the start of treatments) they were sampled and assessed for RCD and the presence of the characteristic structures of *M. bolleyi* on the three first-formed seminal root axes. Other assessments were for: number of tillers, fresh weight of the tops, length of the three first-formed seminal axes, total numbers of seminal and of adventitious roots and over-dry weight (80°C) of the root system, including the three seminal root axes after they had been assessed for RCD.

4.4.1. Results

Pruning of the leaves had a dramatic effect on the growth of the rest of the plant. As seen in Table 4.9, root development practically stopped when or soon after the plants were pruned. The mean length of the first three seminal axes, the dry weight of the laterals that had been excised from these axes and the number of adventitious roots remained almost unaltered thereafter in the pruning treatments. The same dramatic effect was observed for the number of tillers.

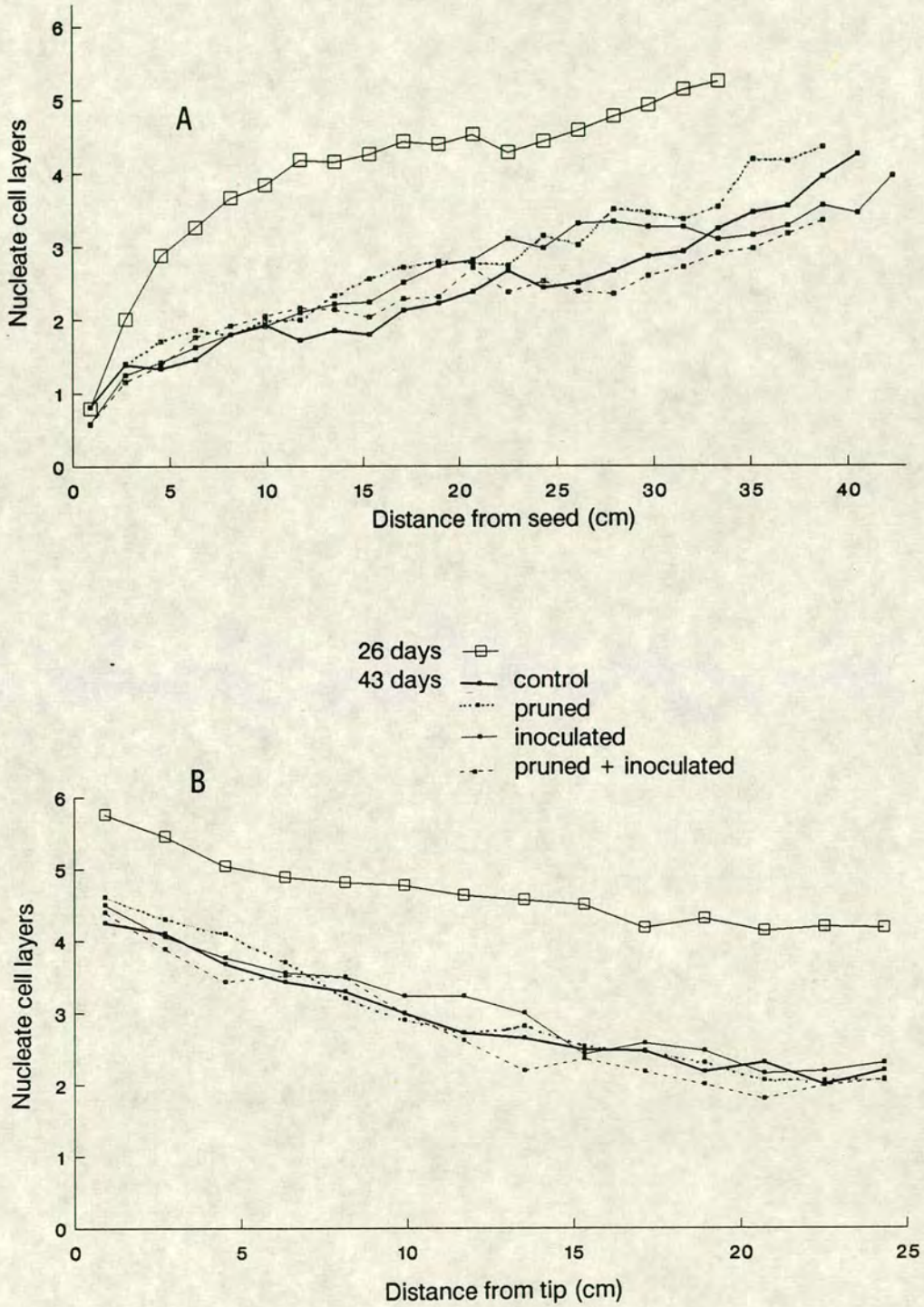
Figures 4.A & B show the amount of RCD along the roots. The results are expressed in different ways in these two figures as

Table 4.9. Effects of pruning of shoots and inoculation of roots with spores of *M. bolleyi* (both initiated after 26 days) on growth of root and shoot systems of wheat plants in tubes of horticultural perlite receiving mineral nutrient solution. Data are means of 8 replicates per treatment.

Treatments	Shoots		Roots				
	Fresh weight (g)	Number of tillers	Length of seminal axes (cm)*	Number of seminal axes	Number of adventitious roots	Total dry weight (mg)	Dry weight of laterals (mg)**
Pre-treatment sampling (26 days)	1.18	0.5	44.2	7.1	1.2	89.3	5.0
Post treatment sampling (43 days)							
Non-pruned, non-inoculated	5.26	2.37	54.6	7.5	7.0	164.9	9.8
Non-pruned, inoculated	4.92	2.25	56.3	6.8	6.6	162.9	9.0
Pruned, non-inoculated	0.82	0.63	45.9	6.9	1.4	84.3	4.5
Pruned, inoculated	0.84	0.88	48.2	7.1	1.2	94.2	4.4
SED	0.22	0.19	2.9	0.3	0.3	8.5	1.0
LSD (5%)	0.45	0.39	5.8	0.6	0.8	17.3	2.0

* Means of three oldest seminal axes on each of 8 replicate plants.
** Mean dry weight of laterals from three oldest seminal axes of 8 replicate plants.

Figure 4.4. Number of nucleate cortical cell layers, after 26 or 43 days, along seminal root axes of wheat seedlings grown in perlite and receiving combinations of pruning and inoculation with *M. bolleyi*; A, assessments based on distance from base of roots (seed); B, assessments based on distance from root tip; means of eight replicate plants per treatment, 3 roots per plant.



explained earlier, to take account of various possible interpretations, because it was not always possible to retrieve root tips and individual replicate roots were of different length. Each point represents the mean of 24 roots (the three first-formed seminal axes from each of eight plants per treatment). As seen in both figures, the amount of RCD increased between the first (26 day) and second (43 day) samplings but at the second sampling there were no gross differences in RCD between the different treatments. This was confirmed by analysis of variance of linear regressions of the data in Fig. 4.4.

Similarly, there were no significant differences between pruned and non-pruned plants in the degree of colonisation of roots by *M. bolleyi*, as assessed by presence of groups of chlamydospore-like structures (results not presented). These were seen in only 2 of the 8 non-pruned plants and 6 of the 8 pruned plants, but interpretation was complicated by the fact that substantial regions of the cortex had sloughed from the roots owing to the age of the plants.

4.5. Experiment 4: effects of reduced nitrate supply and of urea foliar sprays on cortical senescence and susceptibility of wheat roots to take-all infection

Experiment 1 showed that wheat grown with a reduced supply of NO_3-N had an increased rate of RCD. The following experiment was designed to investigate the effect of this on the susceptibility of roots to infection by *Gaeumannomyces graminis* var *tritici* (*Ggt*). With such an experiment the problem always is to identify the effects of different factors under investigation. The supply of nitrogen in the root environment could directly influence the fungus as well as have indirect effects via the host. In order partly to overcome these difficulties an attempt was made to supply nitrogen

to nitrogen-deficient plants by urea sprays applied to the foliage. Because in the earlier experiment a reduction of total nitrogen to one-sixth of that in Hewitt's standard mineral solution caused stunting of the plants, the nitrogen supply in this experiment was reduced to only one-third. Also, root extension rates were recorded daily, in order to overcome problems of comparisons of RCD between roots of different treatments.

Wheat seedlings were sown in perlite in 75 cm long transparent polyethylene tubes, one seed per tube. The tubes were placed at an angle on transparent corrugated perspex frames so that most of the roots grew against the "lower" surface of the polyethylene tube. The corrugated plastic frame had numerous holes in it to provide access for inoculation of roots while the tubes were kept *in situ*. The frame and the tubes, except for their tops, were covered with black polyethylene and aluminium foil to exclude light. Root growth was recorded daily by removing the coverings and marking the positions of root tips on the perspex frame.

The plants were grown in a glasshouse at 20°C under supplementary illumination of 6000 lux; they received, every second day, 200 ml of mineral nutrient solution, and on alternate days 200 ml of distilled water. There were three nutrient treatments as follows.

(1) Hewitt's standard mineral nutrient solution (15 replicates).

(2) Hewitt's mineral nutrient solution but with nitrogen reduced to one third by substituting $CaCl_2$ for $Ca(NO_3)_2$ (20 replicates).

(3) As in (2) above but plants were sprayed with urea when 10 and 17 days old (20 replicates).

The urea spray solution was used at 0.2 M concentration with 0.02% citowett, as a wetting agent. Plants in treatments (1) and (2)

received sprays of water with the wetting agent only. In all cases the plants were sprayed to run-off, and the tops of the tubes were covered with cotton-wool and a foil cap around the bases of the plants until the sprayed solution had dried. A slight "tip burn" was observed on plants sprayed with urea on the first occasion. So the supplementary lights were turned off for 24 h after the second spray, in order to circumvent this effect.

Inoculation of the roots was done when the plants were 15 days old. Four plants from each treatment were left uninoculated and sampled when they were 20 days old. For each of the remaining eleven plants in treatment (1) and sixteen plants in treatments (2) and (3), two roots were inoculated just behind the tips (1 day old regions). The inocula were small agar blocks, 2X2 mm, cut 1cm behind the growing margins of 6-day old PDA colonies of *Ggt* (strain NP2). The inocula were inserted by cutting small windows in the polyethylene tubes and then sealing these again with Sellotape.

When the plants were 25 days old (10 days after the inoculation) they were sampled and assessed, the inoculated roots for invasion and damage from *Ggt* and the remaining seminal roots for RCD. Shoot growth, the lengths of the seminal roots and dry weights of either the whole root system or the laterals removed from the seminal root axes were also recorded.

Before sampling, a detailed map for the age of each part of each individual root visible through the transparent polyethylene was made. Each root was sampled separately and kept in 70% methylated spirits. The inoculated roots were cut at the inoculation point. All seminal roots were assessed for RCD along their length in successive microscope fields. RCD of each part of the root according to its age (days) was obtained with the use of the age-maps. Inoculated roots

were also assessed microscopically for the degree of invasion by *Ggt*, by measuring the length of stelar browning and the numbers of runner hyphae along roots, upwards from the point of inoculation.

4.5.1. Results

Plants receiving nutrient solution of low nitrogen level had significantly reduced shoot growth and root dry weight compared with the plants receiving the standard nutrient solution at both sampling times (Tables 4.10 & 4.11). The effect of low nitrate supply on all parameters of plant growth was compensated for by the urea sprays at the 20 day sampling. But this effect of urea sprays did not persist until the 25 day sampling, when shoot growth was markedly reduced in both "low N" treatments. The reduced N-nutrition had no effect on the production of laterals from seminal roots.

The different nutrient treatments in this experiment had no significant effect on the pattern and rate of RCD. Figure 4.5 shows the mean number of nucleate cell layers of seminal roots in non inoculated plants at 20 days according to age of root regions. A similar pattern was seen if RCD was plotted as a function of root length (not shown). Figure 4.6 shows the pattern of RCD along roots inoculated with *Ggt* and along non-inoculated roots of the same plants. *Ggt* killed the cortical cells only in the areas it invaded. RCD in older parts of the infected roots was not affected. The findings were identical for roots in all nitrogen treatments, so only those for "standard N" are shown.

Table 4.12 presents the invasion of the roots by *Ggt*, assessed as the length of root having brown discolouration and as the length of root from the inoculation point that had runner hyphae. There were no significant differences between the treatments.

Table 4.10. Growth of shoot and root systems of wheat plants grown for 20 days in tubes of horticultural perlite and receiving Hewitt's mineral salts solution with the standard concentration in N, with 1/3 of the concentration and with 1/3 N concentration plus two sprays of urea.*

Treatment	Roots**		Shoots	
	Length (cm)	Dry weight (mg)	Fresh weight (mg)	Dry weight (mg)
Standard	28.7	36.3	282.5	37.6
1/3 N	23.6	24.2	185.0	27.6
1/3 N + Urea sprays	35.7	34.9	302.5	42.7
SED	4.2	3.3	34	4.0
LSD (5%)	9.5	7.5	76.9	9.1

* Means of 4 replicate plants per treatment.

** Mean length of first three seminal axes, and total dry weight of root system.

Table 4.11. Growth of shoot and root systems of wheat plants grown for 25 days in tubes of horticultural perlite and receiving Hewitt's mineral salts solution with the standard concentration in N, with 1/3 of the concentration and with 1/3 N concentration plus two sprays of urea. Two of the first formed seminal roots of each plant were inoculated with Ggt (see text).

Treatment	Roots		Shoots	
	Length* (cm)	Dry weight of laterals*	Fresh weight (g)	Dry weight (mg)
Standard	40.2	3.31	613	91.5
1/3 N	39.8	3.1	335	55.8
1/3 N + Urea sprays	40.0	3.63	396	66.8
SED	5.7	0.35	38	4.4
LSD (5%)			77	8.9

* Mean length (cm) of two first formed, non-inoculated seminal roots, and dry weight of laterals on these roots; 16 replicate plants per treatment (11 plants in standard).

Figure 4.5. Number of nucleate cell layers along seminal root axes of 20 day old wheat seedlings grown in perlite with complete N (standard), N at 1/3 of standard (1/3) or 1/3 N and two sprays of urea. Means of four replicate plants, 3 roots per plant.

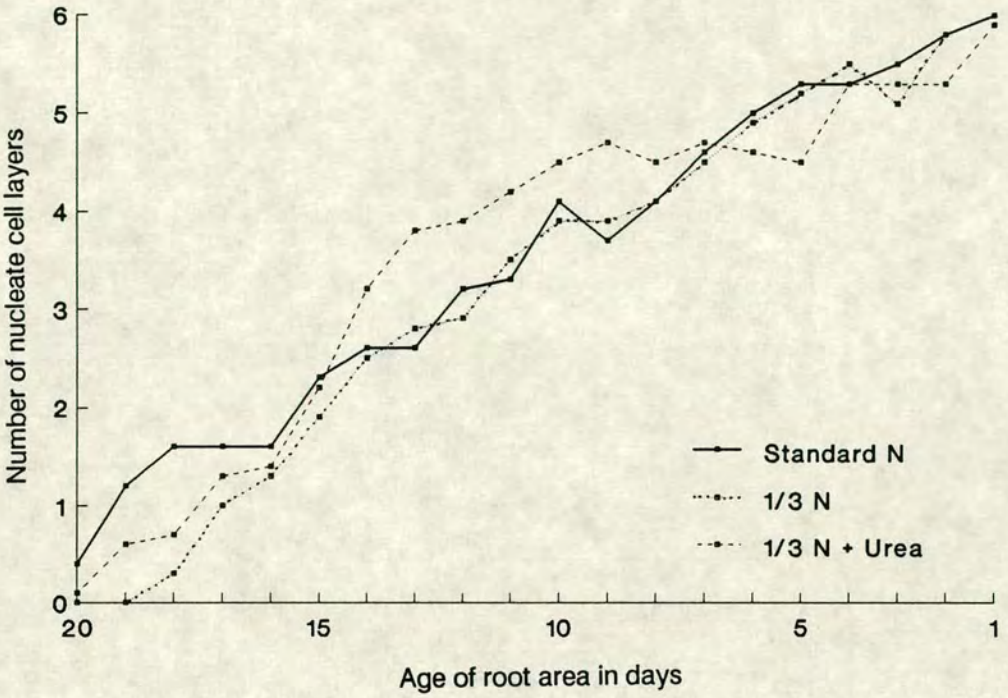


Figure 4.6. Number of nucleate cortical cell layers along seminal root axes of 25 d old wheat plants, grown in complete nutrient solution and either uninoculated or inoculated with *G. graminis* at 11 days at the point indicated by arrow. Data based on 11 replicate plants.

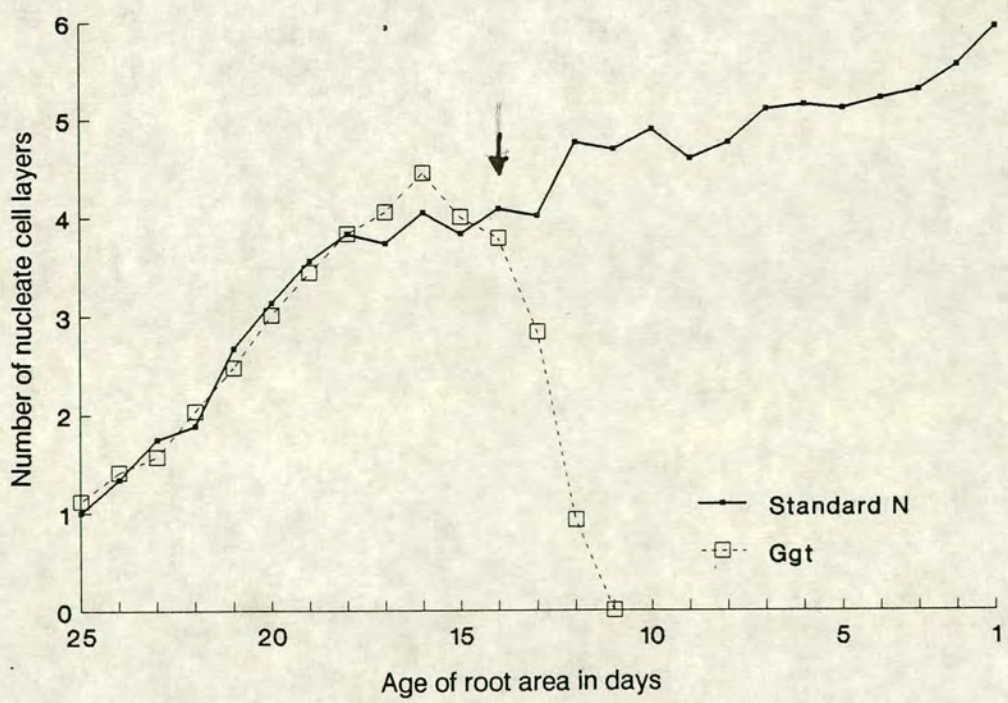


Figure 4.7. Number of runner hyphae along the length of roots upwards from point of inoculation with *G. graminis*. Means of 14 plants (11 for "standard" mineral nutrient treatment).

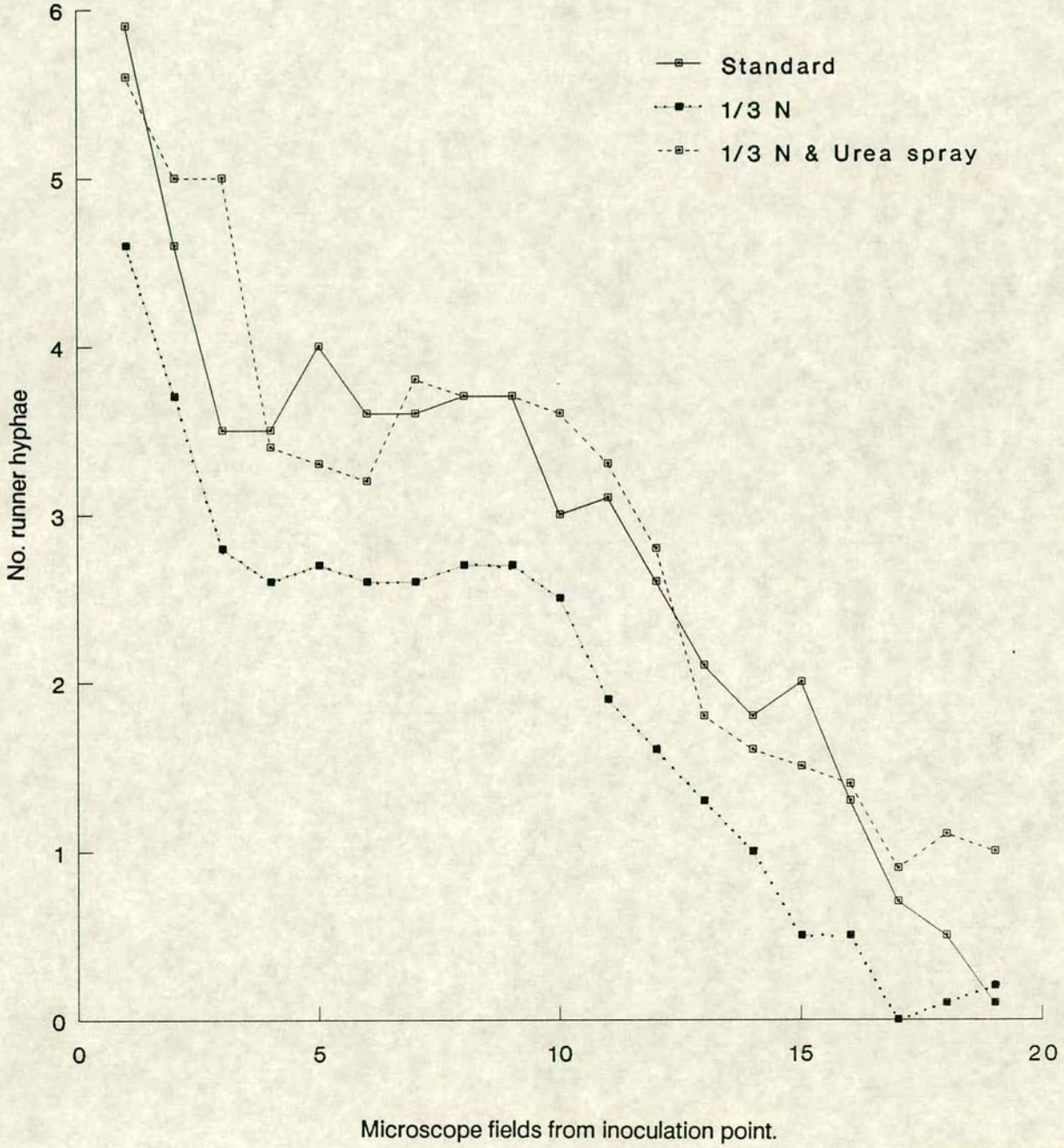


Figure 4.7 presents the number of runner hyphae observed along the root length from the inoculation point upwards, in the three treatments. There were consistently fewer runner hyphae in the low N treatment than in the standard N treatment or "low N + urea sprays".

There were two factors that affected the results in this experiment. Firstly, the differences in N treatments were not sufficient to cause significant differences in RCD in the first-formed root axes. Secondly the amount of inoculum used was perhaps too high to lead to major differences in establishment of infection. The inoculated roots stopped growing at about 4-6 days after inoculation, irrespective of differences in mineral nutrition of the plants.

Table 4.12. Extent of symptom development upwards from inoculation point and subsequent growth of root after inoculation in seminal roots of wheat plants grown in perlite with complete nutrient solution, with N reduced to 1/3 of the standard or with 1/3 N plus two sprays of urea; all inoculated with *G. graminis* *

Treatment	Length of root with runner hyphae (mm)	Length of root showing browning (mm)	Growth of root after inoculation (mm)
Standard	25.4±1.4	22.0±2.3	110±10
1/3 N	22.1±1.1	17.1±1.3	97± 9
1/3 N & urea sprays	25.9±1.8	20.7±1.8	109± 7

* Means and SE of 16 plants per treatment (11 plants in standard).

4.6. Experiment 5: senescence of tomato roots and disease development by *Pyrenochaeta lycopersici*

P. lycopersici and glasshouse-grown tomato plants were chosen as a host-parasite system to study relationships between the development and senescence of roots in a dicotyledonous plant and disease caused by invasion of the cortex by a fungus.

In preliminary experiments it was observed that tomato seedlings inoculated with spores of *P. lycopersici* did not develop symptoms until they were 30-35 days old. The first symptoms developed in the older roots, including the tap root and older laterals and the adventitious roots that emerged later from the base of the stem, grew fast and underwent secondary thickening. These observations provided the basis for this and the following experiment (6) to be described later.

Surface-sterilised tomato seeds were pre-germinated for 2 days on sterile moist filter paper in Petri dishes in darkness at 25°C. Eight such seeds were placed in each 200 ml perforated plastic cup filled with perlite. Seeding was staggered at 10 day intervals over 30 days, with 30 cups at each time, to give three age groups of plants. The experiment was done in a glasshouse at 20±1 °C, without supplementary illumination. The cups were placed in transparent plastic propagators until the seedlings had emerged; then they were irrigated every second day with 50 ml of Hewitt's mineral solution. Following emergence, the plants were thinned to 6 per pot. When plants in the three age groups were 10, 20 and 30 days old, half of the cups of each group were inoculated with 200 ml of a spore suspension of *Pyrenochaeta lycopersici* in mineral nutrient solution (10^4 spores per ml) added to the top of each cup and allowed to drain through.

Twenty five days after inoculation, 6 cups per age group (and 6 non-inoculated, control cups) were sampled; the remaining cups were sampled 39 days after inoculation. The root systems were assessed as follows.

1. Visible symptoms of disease, expressed as percentage of the total root length. For this, the root system was washed from perlite, spread in a thin layer of water in a Petri dish and an intersection method was used to estimate the total root length and the percentage of this that was brown or obviously dead. In an attempt to relate the distribution of symptoms to root age, the diameter of roots on the intersect lines was also recorded, but only in the second sampling when symptoms were more advanced than in the first.

2. The tap root and 3-4 laterals (taken at random) from roots of plants in the first sampling were washed, cut into 5 mm pieces and plated on medium selective for *P. lycopersici* to record the percentage of root pieces from which *P. lycopersici* emerged.

3. Roots from plants in the second sampling were cut into 5 mm pieces and surface sterilised in a saturated solution of calcium hypochlorite for different times before being plated on selective medium. The effects of age at inoculation and time of surface sterilisation on recovery of *P. lycopersici* were determined for roots of different diameter and root pieces with and without disease symptoms.

4.6.1. Results

The isolate of *P. lycopersici* used in these experiment was of high pathogenicity and when small agar blocks or spores were placed on roots of young seedlings, they were completely destroyed in a few days (see Section 3.11). The development of disease symptoms,

however, in the glasshouse-grown plants was slow and much like that in commercial practice (Last & Ebben, 1966).

No symptoms of root damage were observed in roots of non-inoculated (control) plants, and no colonies of *P. lycopersici* or other grey sterile fungi emerged when these roots were plated on selective medium.

The development of symptoms in the three age-inoculation groups of plants is shown in Table 4.13. When the plants were sampled 25 days after inoculation, few symptoms had developed and there were no differences in the extent of symptoms between the different age-inoculation treatments. In an attempt to estimate the degree of colonisation by the fungus, roots were plated on selective medium. Table 4.14. shows that there were no significant differences between treatments, and although the roots had developed symptoms on only 2-3 percent of their length, the fungus seemed to be present on about one third of the root length in all treatments.

Roots of plants in the second sampling had more symptoms (Table 4.13) and there were now differences between age-inoculation groups: the plants that were older at inoculation had significantly more roots diseased than in the two other treatments ($P<0.001$). Moreover, for these older plants Table 4.15 shows that 90% of the brown (diseased) roots were in categories of root diameter 0.4 mm or larger, although these wide (and presumably older roots) presented only 17.8% of the total root length.

Figure 4.8 shows the effect of surface sterilisation on recovery of *P. lycopersici* from root pieces with and without symptoms, the roots being taken from plants inoculated when 30 days old and sampled 39 days later. No reduction in the recovery of the fungus was found with increasing time of surface-sterilisation of brown

Table 4.13. Percentage of root length showing symptoms (browning) in tomato seedlings inoculated with spores of *P. lycopersici* when they were 10, 20 or 30 days old and sampled 25 (first sampling) and 39 days (second sampling) after inoculation.

Age of plants at inoculation (days)	Percentage (as arcsine) of root length with symptoms*	
	First sampling	Second sampling
10	2.6 ± 0.7	4.6 ± 1.5 a
20	1.7 ± 0.3	7.0 ± 1.2 a
30	2.8 ± 1.1	16.5 ± 2.0 b

* Means of 6 replicate cups in the first sampling and 9 in the second sampling. Figures in the second column followed by different letters differ significantly ($P<0.001$).

Table 4.14. Percentage* of 5 mm pieces of tomato roots from tomato seedlings inoculated when they were 10, 20 and 30 days old and sampled 25 days later that yielded *P. lycopersici* when plated on selective medium; tap roots and laterals assessed separately.

Age of plants at inoculation (days)	Tap roots	Laterals
10	29.9 ± 4.3	20.5 ± 3.4
20	37.9 ± 3.9	31.5 ± 4.0
30	23.4 ± 3.9	34.6 ± 6.3

* Means and SE of 6 replicate cups, data transformed to arcsine.

Table 4.15. Relationship between root diameter and symptoms of brown root rot caused by *P. lycopersici* in plants inoculated when they were 30 days old and sampled 39 days later.

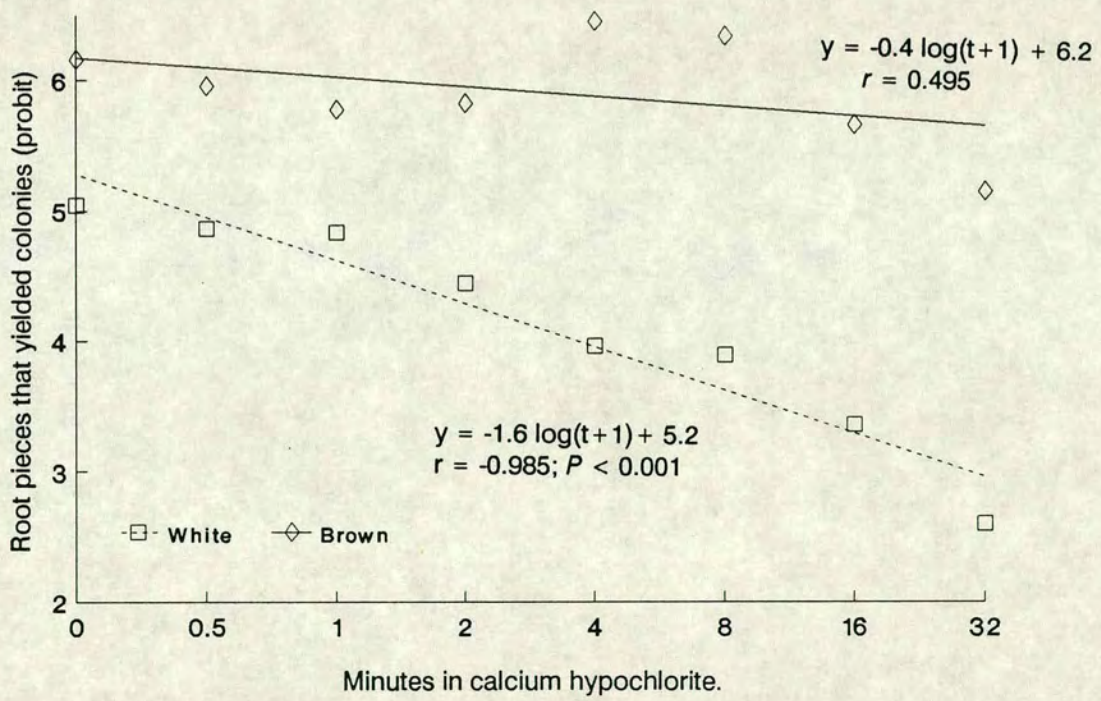
Root diameter (mm)	Number of root intersections assessed	Percent of total root length	Number of intersections with symptoms	Percent length with symptoms
0.2-0.3	541	50.2	16	3 a
0.3-0.4	346	32.1	32	9 b
0.4-0.5	116	10.8	28	24 c
0.5-0.6	62	5.8	22	35 c
> 0.6	13	1.2	4	31 c

Percentages in last column followed by different letters differ significantly (P=0.05).

Table 4.16. Relationship between root diameter and percent of root pieces yieding *P. lycopersici*, from plants inoculated when they were 30 days old and sampled 39 days later. Data for roots with no visible symptoms, pooled for all surface-sterilisation times.

Root diameter (mm)	Percent root pieces	Percent divided by mean diameter
0.2-0.3	14.5	5.8
0.3-0.4	23.5	6.7
0.4-0.5	26.5	5.9
0.5-0.6	30.0	5.5
0.6-0.7	36.0	5.5
> 0.7	40.8	5.2

Figure 4.8. Log (dose)- probit curves of the effect of different times of surface-sterilisation on the recovery of *P. lycopersici* from brown roots and white, symptomless roots of tomato plated onto a selective medium; data from 69 day old plants inoculated with the fungus 39 days previously.



(symptomed) roots. However, there was a significant progressive reduction in recovery from white, symptomless roots, with increasing time of surface-sterilisation. Evidently the fungus was present mainly on the surface of these roots.

Pooled across all times of surface-sterilisation, there was an apparent increase in percentage of the root pieces without symptoms that yielded *P. lycopersici* with progressive increase in diameter of the roots (Table 4.16). However, when these percentages were divided by the root diameter, there was no such difference, suggesting that the relationship was due solely to chance - a larger root volume giving more probability of the fungus surviving any given surface-sterilisation treatment.

As disease symptoms were seen mainly on older plants and in the widest (and presumably oldest) roots (Tables 4.13 & 4.15), it was of interest to examine cortical senescence in equivalent uninoculated plants. This was done with the neutral red-plasmolysis method, which revealed that most of the cortical cells were alive, although much of the root epidermis was dead. There was thus no basis for explaining the incidence of disease in terms of natural senescence of the root cortex.

4.7. Experiment 6: effect of age of tomato plants on infection by *P. lycopersici*

This experiment stemmed from the findings of Experiment 5, above because it was recognised that the development of browning symptoms in older parts of tomato root systems could have been related to the build-up of inoculum in older root regions due to the high root density there. Cups of perlite, as before, were sown with tomato and the plants were allowed to grow for two weeks. Then further seeds

were sown at intervals so that each cup would contain plants of different ages. Spore inoculum was then added to the cups, when there were one, two and four weeks old plants in the same pot and six weeks later, the plants were assessed for root browning systems.

Unfortunately, there were numerous technical difficulties in this experimental design, which complicated interpretation of the results or even the collection of full results. The young plants grew very poorly in the presence of older plants, and the root systems were so entangled that they could not be separated from one another. Nevertheless, it was clear from the parts of the root systems that could be identified as belonging to individual plants that root browning symptoms were as common on the youngest (seven week) plants as on the much larger ten week-old plants. Representative results to illustrate this are shown in Table 4.17.

4.8. Further studies on browning root of tomato

Several further experiments with tomato roots, were designed to investigate the relationships between root age, cortical senescence and development of *P. lycopersici*. All presented technical difficulties of some sort, the basic problems being the lack of a well-defined pattern of root development, the generally low level of cortical senescence and the difficulty of retrieving complete root systems, especially the ephemeral and easily damaged terminal rootlets. However, two related experiments gave worthwhile results and the relevant parts of them are described below.

Both experiments involved growing two plants in a cup of perlite moistened with complete mineral nutrient solution as before. The plants were of the same age but one of them was subsequently decapitated at soil level in an attempt to hasten root cortical

Table 4.17. Percentage of root length showing symptoms (browning) in tomato plants of different ages growing in the same cup and inoculated with spores of *P. lycopersici* when they were 1, 2 or 4 weeks old and sampled 6 weeks after inoculation.

Age of plants at sampling (weeks)	Percentage (as arcsine) of root length with symptoms	
	Tap root	Laterals
7	25 ± 4.2	27 ± 4.5
8	20 ± 5.7	23 ± 3.4
10	19 ± 4.2	22 ± 5.8

Table 4.18. Effect of root inoculation with spores of *P.lycopersici*, and of decapitation, on mean number of dead root cortical cell layers of tomato plants derived from cuttings.*

Sampling (Days after shoot removal treatment)	Removal of shoot	Inoculated	Non-inoculated	Row mean
10	Yes	0.85	1.12	0.98
	No	1.11	0.93	1.02
20	Yes	1.58	1.10	1.33
	No	1.21	1.17	1.19
30	Yes	2.21	1.44	1.82
	No	0.83	1.00	0.91
Column mean	Overall	1.30	1.12	1.21
	Yes	1.55	1.22	1.38
	No	1.05	1.03	1.04

* Means of 3 replicate plants (5 replicates for day 10 sampling). SED for row means (underlined), 0.23; LSD 5% = 0.47; inoculation had no significant effect.

death. In one experiment the plants were derived from seed; in the other they were raised from cuttings in the cups of perlite so that they had several relatively uniform adventitious roots.

Seed-derived plants were grown for 20 days then inoculated by drenching the cups with spore suspension of *P. lycopersici* (10^4 spores ml^{-1}) as described earlier. At 30 days (10 days after inoculation) the shoot was removed from one of the two plants in each cup. Uninoculated control plants, with and without decapitation, were included for comparison. Four replicate cups were sampled when the plants were 35 days old, and four when they were 40 days old, before the experiment was abandoned for technical reasons. At sampling, the roots of the plants in each pot were carefully separated and representative samples were cut into 5 mm lengths, surface-sterilised for 2 min in calcium hypochlorite solution and plated on selective medium. None of the plants had developed root symptoms when sampled 15 or 20 days after inoculation with *P. lycopersici*. But the fungus was recovered from many root pieces that were plated onto selective medium - 27% of those from the "intact" plants at both samplings, compared with 41% and 53% of roots from the decapitated plants at 15 and 20 days after inoculation. The difference between intact and decapitated plants was significantly different ($P<0.05$) by paired-samples analysis.

The experiment with cuttings was of identical design except that decapitation was done at 20 days when the plants were inoculated, and the plants were sampled when 30, 40 and 50 days old (10, 20 and 30 days after inoculation). Again there were two plants per cup, one with the shoot removed and one left intact. None of the plants developed browning root symptoms but they were used to assess root cell viability by neutral red-plasmolysis using an intersection method.

The most striking feature of this experiment was the longevity of roots of decapitated plants. Thirty days after removal of the shoot, most of the cortex was still alive, even in roots inoculated with spores of *P.lycopersici* (Table 4.18). There were, however, small but significant differences in root cell death between treatments: removal of the shoot increased the mean number of dead cortical cell layers from 1.0 to 1.4 ($P < 0.02$) when results were pooled for inoculation treatments and different sampling times. Inoculation with *P. lycopersici* also increased the mean number of dead cortical cell layers, but only in the decapitated plants.

Table 4.19. Effect of inoculation with spores of *P.lycopersici*, and of decapitation, on mean diameter (mm) of roots sampled 10, 20 and 30 days after the decapitation and inoculation treatments. Data for tomato plants derived from cuttings.*

Sampling (Days after shoot removal)	Removal of shoot	Inoculated	Non-inoculated	Row mean
10	Yes	0.36	0.38	0.37
	No	0.32	0.33	0.32
20	Yes	0.38	0.43	0.41
	No	0.29	0.36	0.33
30	Yes	0.40	0.44	0.42
	No	0.30	0.35	0.32
Column mean	Overall	*0.34*	*0.38*	0.36
	Yes	0.38	0.42	<u>0.40</u>
	No	0.30	0.35	<u>0.33</u>

* Means of 3 replicate plants (5 replicates for 10 day sampling). Top removal and inoculation affected significantly ($P<0.001$) the mean root diameter (underlined and italicised means respectively).

The mean diameter of roots in different combinations of treatments and samplings is shown in Table 4.19. It remained constant with time in the intact plants, but increased progressively with time in the decapitated plants, the diameter (pooled for all times) being significantly (P=0.001) greater for decapitated than for intact plants. This is most easily explained by assuming that the finer roots were progressively lost, by decay, in the decapitated plants. Roots of plants inoculated with spores of *P.lycopersici* had a highly significant (P<0.001) decrease in mean root diameter compared with that in uninoculated plants. This was true irrespective of the removal of the shoots. It is difficult to explain because the fungus would be expected to kill the finer roots.

4.9. Discussion.

The work in this section supports previous reports of the pattern of RCD in cereals. In all experiments fluorescing nuclei disappeared first from the root epidermis and then from successively deeper cortical layers with increasing age of the root regions. Nuclei tended to persist, but only temporarily, in many cell layers of the root axes around the bases of the laterals, and nuclei often persisted for a considerable time in the innermost cortical cell layer, next to the endodermis, after they had disappeared from other cell layers of the cortex. These results were in agreement with the experiments in Section 3, where excised root pieces were used.

The environmental variables used in this work did not influence the general pattern of RCD but had, in some instances, major influences on the rate of cell death. Reductions of soil nitrogen and calcium from apparently near-optimum levels were found significantly to increase the rate of RCD. This is the first such

report for calcium but Gillespie & Deacon (1988) previously showed that deficiency of nitrogen or phosphorus caused an increase in the rate of RCD of wheat. Brown & Hornby (1987) also found that reduced nitrogen increases the rate of RCD in gnotobiotic conditions. The effects of nutrient deficiencies were seen as an acceleration of cell death along the whole length of roots, which still seemed to be functional because the root tips continued to grow. However, a distinction must be made between different degrees of nutrient deficiency in this respect. At the lowest levels of Ca^{2+} plant performance was severely affected and the roots showed evident dysfunction. The root tips of severely calcium-deficient plants were brown and effectively ceased growing. Such treatments were excluded from analysis of the effects of mineral nutrients on RCD. Probably an absence of N would also have caused major dysfunction of plants. Conversely, relatively low degrees of mineral deficiency such as 1/3 N in Experiment 4 and 1/3 Ca in Experiment 2 had no measurable effect on RCD despite the fact that shoot growth was markedly reduced by such treatments. The effects of N deficiency on RCD were seen with 1/6 N in Experiment 1 and the effects of Ca deficiency on RCD were seen with 1/9 Ca in Experiment 2. In explanation of all these findings it is proposed that severe mineral nutrient deficiency affects shoot growth or shoot function so much that the roots are effectively starved of photosynthate. Less severe deficiency affects shoot growth and thus the production of new roots but still enables the existing roots to be maintained without accelerating their cortical senescence because a proportion of the photosynthate still reaches these roots. Thus we see the apparent anomaly that there was no increase in RCD (of the existing roots) at one-third of "standard" mineral nutrient levels even though shoot

growth was markedly affected by such treatments.

The same explanation was proposed by Lewis & Deacon (1982) to account for the failure of shading or powdery mildew infection of cereals to influence RCD even though total root growth and shoot growth was markedly reduced by these treatments. Shading and pruning of the shoots of wheat also did not affect RCD in the experiments reported here, and yet these treatments markedly reduced both root and shoot growth. Shoot pruning, for example, almost immediately stopped any further root growth. The explanation of Lewis & Deacon (1982) might apply. In support of it are the results in Section 3.5 (and of other workers) which show that viability of the cortex of detached cereal roots is influenced by the supply of sugars; so an hypothesis linking the rate of RCD in whole plants to assimilate supply is not unreasonable. An alternative hypothesis is that the rate of RCD in existing roots of whole plants is largely governed by the energy reserves in the cortical cells which may not receive much assimilate once the root tip has grown on. The rate of RCD in these conditions would then be independent of the supply of photosynthate and thus independent of factors that affect shoot growth. Yet another possibility is that roots can recycle the components of senescing cells such that the death of some cells from natural senescence helps to maintain the viability of other cells. A major recycling of phosphorus from senescing cells in cereal roots was proposed by Robinson (1990), who argued that wheat seedling roots, beyond a certain age, could become largely independent of soil-derived phosphorus if this is internally remobilised from naturally senescing cells. Such an hypothesis is equivalent to the withdrawal of materials from leaves before leaf fall - a parallel that was noted by MacLeod *et al.* (1986).

It is compatible with the finding that rate of RCD in roots attached to seedlings or detached from them is influenced by the levels of available mineral nutrients. In other words, RCD might be part of a regulatory process whereby mineral nutrients are remobilised when in short supply. Studies on detached root pieces (Gillespie & Deacon, 1988) have shown that RCD is affected directly by the availability of mineral nutrients supplied at the root surface.

In all these respects it must be said that interpretation of experimental results is very difficult. A distinction needs to be made between direct effects of treatments on roots (or RCD) and indirect effects operating through the shoot system or the plants' hormonal balance. Also, it is not clear whether RCD in detached roots should be directly equated with that in roots attached to plants. Different explanations may apply to the effects of mineral nutrient and photosynthate supply, because roots normally obtain mineral nutrients from their immediate surroundings -the soil- whereas their supply of energy comes from the shoot system. There is urgent need to address all these issues, including the possible remobilisation of nutrients, in studies with radioactive tracers. Such studies should also address the differences in behaviour between cereals and dicotyledonous plants such as tomato.

One of the most remarkable features of the work in this section was the observed longevity of tomato root cortical cells, even on plants that were incubated for 30 days in soil with their shoots removed. This supports and extends the work in Section 3.3, where it was shown that detached tomato roots could maintain full viability of the cells if only a very low level of sugar was supplied in the supporting medium. Ever more notable was the finding that

inoculation of such decapitated plants with *P. lycopersici* did not cause a large increase in rate of root cortical death. Such experiments could usefully be repeated, using higher inoculum levels of the fungus because the provisional findings from the experiments on tomato plants indicate that seedlings can succumb to cortical infection if grown in the presence of older plants, when the combined root density is high and the fungus may thus establish a high inoculum level (and associated food reserves) from which to infect.

Rush *et al.* (1984) studied the relationship between root cortical sloughing and development of foliar symptoms in cotton infected by *Phymatotrichum omnivorum*. They showed that symptom development above-ground occurred only after the root cortex was sloughed as a result of infection by the fungus. The relationship of this to natural root senescence, if any, remains unknown. It may be that cortical senescence as such is uncommon in dicotyledonous plants except when their roots undergo secondary thickening. More important in terms of the development of pathogen populations may be the apparently normal shedding of the terminal fascicles of roots, including the fine "feeder roots". This process was described in detail by Wilhelm & Nelson (1970) for strawberry plants. The natural "feeder root turnover" was accelerated by weakly parasitic fungi. Evidence of a similar phenomenon was found in this section, because the mean root diameter of tomato plants was increased by removal of the shoots. However, for unknown reasons, inoculation of plants with *P. lycopersici* had the opposite effect. In many of the experiments it was recognised that the terminal branches of tomato root systems were not recovered from perlite. Some of these terminal feeder roots are extremely fine and they seemed to be rotted even in control

(uninoculated) plants. In future work it would be advantageous to use borescopes (Rush *et al.*, 1984) or video techniques with transparent tubes containing perlite to follow the patterns of root turnover, as was done for apple trees in a root observation laboratory (Atkinson, 1974).

Colonisation of wheat roots by *M. bolleyi* was enhanced by applying the spore inoculum together with glucose. This showed that spores even in the high concentrations used in these experiments need some external energy resource in order to colonise the root cortex effectively. Colonisation of wheat root cortices by *M. bolleyi* was also increased in treatments where plants showed more RCD as a result of calcium deficiency but was unaffected by leaf pruning, which did not affect RCD. This provides some support for the view that *M. bolleyi* exploits senescing cortices (Kirk & Deacon, 1987b). However, the estimate of the degree of colonisation of the root cortex by *M. bolleyi* was provided by the number of characteristic chlamydospore-like structures seen in the root cortex. This method of assessment probably detects only gross differences in establishment by the fungus; it does not give information on the extent to which the fungus is present in the root cortex as hyaline mycelium. This perhaps explains why there was not always a clear relationship between RCD and establishment by the fungus. For example, there was no significant difference in establishment (assessed as groups of chlamydospore-like structures) across the range of calcium treatments in Experiment 2 when spores were applied without sugar, despite major differences in RCD. Conversely, there was better establishment by *M. bolleyi* (supplied with sugar) at the standard Calcium level compared with twice this level (Table 4.8) and yet there was no difference in RCD between

these treatments. According to the hypothesis of Deacon & Henry (1980) for *G. graminis* and of Kirk & Deacon (1987) for *M. bolleyi*, RCD is most significant in enabling fungi to increase their populations from very low initial levels or to maintain themselves at low levels (giving inapparent infections) rather than in leading to the establishment of massive levels of infection the equivalent of which, for *M. bolleyi*, would be the development of dark structures in the roots. Although *M. bolleyi* causes little damage to roots, it did slightly enhance the rate of RCD at the inoculum levels used in these experiments, as in root pieces in Section 3. This contrasts with the findings of Henry & Deacon (1981) and Kirk & Deacon (1987) but it is compatible with other reports of minor damage caused by *M. bolleyi* (Fitt & Hornby, 1978; Stetter & Leroul, 1979).

The effect of low nitrogen level on rate of RCD may help to explain the common observation that take-all infection is most severe at low nitrogen levels. A high rate of RCD is indicative of declining resistance of cortical cells to invasion and will also boost the inoculum level of the pathogen by supplying carbon nutrients (Deacon & Henry, 1980; Deacon, 1981).

Nitrogen nutrition has major effects on take-all of cereals, as reviewed by Huber (1981). Nitrogen deficiency in agricultural soils is associated with severe outbreaks of take-all, this effect having been noted since the late 1800's and repeatedly confirmed. Part of the beneficial effect of nitrogen fertilisers is undoubtedly to enable root production and thereby offset damage to the existing roots caused by *G. graminis* var. *tritici* (Glynne, 1953; Garrett, 1970). However, various workers have shown more direct effects of nitrogen in increasing or decreasing root infection by the take-all fungus (reviewed in Huber, 1981).

An attempt was made to differentiate between these effects of nitrogen by using urea foliar sprays. There is evidence that such foliar sprays can affect the nature and amount of root exudates and so affect rhizosphere population. Agnihotri (1964) found that there was an increase in concentration of amino acids and a decrease in concentration of organic acids in root exudates of urea-treated wheat plants. There were also differences in the *Aspergillus* spp. isolated from treated and non-treated plants. Unfortunately, the major experiment designed to investigate effects of urea sprays in this section suffered from several inadequacies which would not easily be overcome without making such experiments highly artificial. *Ggt* rapidly invaded and killed roots from even small inocula. Also, the rate of RCD was not affected by a level of N deficiency that markedly reduced shoot growth and so urea sprays did not affect the rate of RCD. But nitrogen supply did have a small effect on *Ggt* in Experiment 4 because there was a consistent reduction in the number of runner hyphae along roots in the low N treatment compared with in both the "standard N" treatment and "low N plus urea sprays".

There are several reports of increased take-all after application of calcium (Huber, 1981 and 14 references therein). Although calcium is usually applied to increase soil pH rather than as a fertiliser, it is also important in the physiology of the host and presumably of the pathogen. There is evidence of an effect of calcium on take-all disease independent of its effect on soil pH. *Ggt* is known to grow satisfactory over a wide range of pH (Garrett, 1956), but outbreaks of take-all occur in response to relatively small pH changes after liming (Smiley and Cook, 1971, 1973; Smiley, 1975). In addition, increased take-all was observed when either

calcium sulphate (neutral salt as gypsum) or calcium carbonate (lime) were applied to soil, suggesting an effect independent of pH (Huber, 1981). The mechanisms by which these calcium effects occur are not known. Low calcium has here been shown to affect RCD when the levels of calcium were severely inhibitory to shoot growth. It is unlikely that this is relevant to the field studies mentioned above. Potentially more relevant is the finding that calcium at supraoptimal levels for shoot growth could reduce the establishment of infection by *M. bolleyi* (Experiment 2). This fungus is a known biocontrol agent of take-all (Kirk & Deacon, 1987a) so any reduction in its establishment might benefit *Ggt*. But further work is needed to determine how calcium supply affects *Ggt* in equivalent conditions.

In summary of the findings in this section, it is recognised that RCD is only one of many interacting factors that can affect the behaviour of root-infecting fungi. It is recognised also that treatments that affect RCD may not have a simple, direct mode of action but may operate through assimilate partitioning in plants and through other physiological processes. All these points make it difficult to draw firm conclusions about the relationship between RCD and the growth of pathogens or other root parasites. In terms of symptom expression above-ground, it is perhaps more relevant to consider the effects of mineral nutrient deficiency, shading, etc, on root growth *per se* rather than on relatively small differences in root cortical senescence. However, it may be relevant to consider RCD in terms of the early establishment of fungi on roots and the build-up of their populations to potentially damaging levels or to levels at which they may exert biocontrol.

Manipulation of the rate of RCD by experimental treatments potentially provides a means of studying such effects. Unfortunately, the experiments in this section demonstrate that relatively harsh treatments need to be applied in order to achieve even small differences in rates of cortical senescence, and such harsh treatments have much more pronounced effects on other plant processes which complicate interpretation of the findings.

5. Seed inoculation with *Microdochium bolleyi* and sporulation of the fungus in the spermosphere and rhizosphere of wheat

5.1. Introduction

Experiments in this section concerned seed treatment as a means of introducing biocontrol agents into the rhizosphere, and study of sporulation on germinating seeds and on roots as a basis for dispersal of *M. bolleyi* in the rhizosphere.

Usually a large amount of inoculum must be incorporated into soil in order to achieve effective biocontrol of a disease. Seed treatment is an attractive alternative method for introducing biocontrol agents into the rhizosphere, because it is practicable and of low cost. However, if seed treatment is to be effective (a) a reasonable amount of the inoculum must survive after application to the seed, (b) the inoculant organism must colonise the seed and other parts of the developing seedling, using energy resources to increase its inoculum level and at the same time depriving pathogens from using these resources, and (c) the biocontrol agent must spread downwards and rapidly colonise the rhizosphere.

Seed treatment has been studied with bacterial biocontrol agents (Merriman *et al.*, 1974; Burr *et al.*, 1978; Kommedahl & Windels, 1978; Windels & Kommedahl, 1978) and with fungal biocontrol agents (Gregory *et al.*,1952; Liu & Vaughan, 1965; Harman *et al.*, 1981; Hubbard *et al.*, 1983; Haddar *et al.*, 1984; Chao *et al.*, 1986), for crops such as lucerne (Gregory *et al.*, 1952), maize (Chang & Kommedahl, 1968; Kommedahl & Mew 1975; Kommedahl *et al.*, 1981), cotton (Morrow *et al.*, 1938), table beet (Liu & Vaughan, 1965), peas (Kommedahl *et al.*, 1981; Hubbard *et al.*, 1983), soybean (Kommedahl *et al.*, 1981) and cucumber (Paulitz *et al.*, 1990).

The results of these studies show that the nature, quality and quantity of the inoculum play important roles in the success of the method. Spores applied to seeds must germinate when the seed is placed in soil, overcoming soil fungistasis. This usually happens because the germinating seed and the developing root create an environment rich in nutrients (spermosphere and rhizosphere respectively) which annuls fungistasis. For example, conidia of *Penicillium oxalicum* Currie & Thom can germinate on the seed coat within 3 days after the seed is planted in soil (Windels, 1981). The physiological state of the spores is important, as shown again for *P. oxalicum* because spores produced on PDA were less effective as inoculum for seed treatment than spores produced on Czapek or malt agar (Kommedahl *et al.*, 1981). The same authors observed that fungal biocontrol agents applied to seeds had colonized pericarps or seed coats of corn, peas or soybean, and could be recovered from roots of 3-8 week old seedlings. However, little information exists regarding the movement of microorganisms from seed to the rhizosphere. An effective biocontrol agent must be able to colonize the rhizosphere, and this can be a major limitation to success of a seed-applied inoculum. Among various reports, organisms applied to seed were recovered from the rhizosphere for only a short time (Kerr, 1961; Mitchell & Hurwitz, 1965), at only short distances from the seed (Chao *et al.*, 1986), in low numbers (Merriman *et al.*, 1974), were not recovered but affected other organisms in the rhizosphere (Kommedahl *et al.*, 1981) or were effective colonisers of the rhizosphere (Kloepper *et al.*, 1980).

Papavizas (1982) reported that *Trichoderma harzianum* Rifai did not survive well in the rhizosphere of bean and pea seedlings when seeds were coated with conidia of the fungus. In contrast, Ahmad &

Baker (1988) have reported that mutants of *T. harzianum* have enhanced "rhizosphere competence" assessed as the ability to colonise roots of seedlings in perlite. Chao *et al.* (1986) found that antagonism by the soil microflora may prevent fungi applied to seed from colonizing the developing roots, but such colonisation occurred when seeds were sown in sterile soil. Also, they found that percolating water enhanced the downward movement of both bacterial and fungal propagules in the rhizosphere. Bahme & Schroth (1987) have similarly shown that colonisation of the potato rhizosphere by tuber-applied fluorescent pseudomonads is greatly facilitated by irrigation in field soils.

Any downwards spread in percolating water will be much faster than by mycelial growth of fungi and could potentially enable a fungus to keep pace with the rate of root extension, which for cereals such as wheat can exceed 3 cm day^{-1}. Taylor & Parkinson (1961) measured the growth of fungi along roots of broad beans as 3 mm day^{-1}, much slower than that of the root (9mm day^{-1}), and they concluded that colonisation of roots from soil was more significant for young root surfaces than was migration of existing fungi along the root. But downwards spread in percolating water requires that a fungus produces movable propagules from established foci of colonisation on the seed or older root regions, and there is little information about this.

Among the fungi often isolated from roots of cereals and grasses, *G. graminis* and *P. graminicola* do not, apparently, form dispersible spores in the rhizosphere. Consistent with this is the fact that their dark runner hyphae can be traced continuously from initial foci of infection to the furthest extent of root colonisation. However, *M. bolleyi* sporulates readily on agar media

and also in liquid culture media, and it also can produce secondary spores directly from germinating spores without the formation of germ-tubes (section 6). So, in view of the potential role of this fungus as a seed-applied biocontrol agent of take-all (Kirk & Deacon, 1987a), a series of experiments was done to assess the potential for colonisation of wheat roots by spores of this fungus, derived initially from seed- or root-applied inocula.

5.2. Experiments 1: spore production by *M. bolleyi* from seed-inoculated wheat

Wheat seeds (cv Avalon) were surface sterilised (10 seconds in 95% ethanol then 20 min in a saturated aqueous solution of calcium hypochlorite) then washed thoroughly in sterile distilled water.

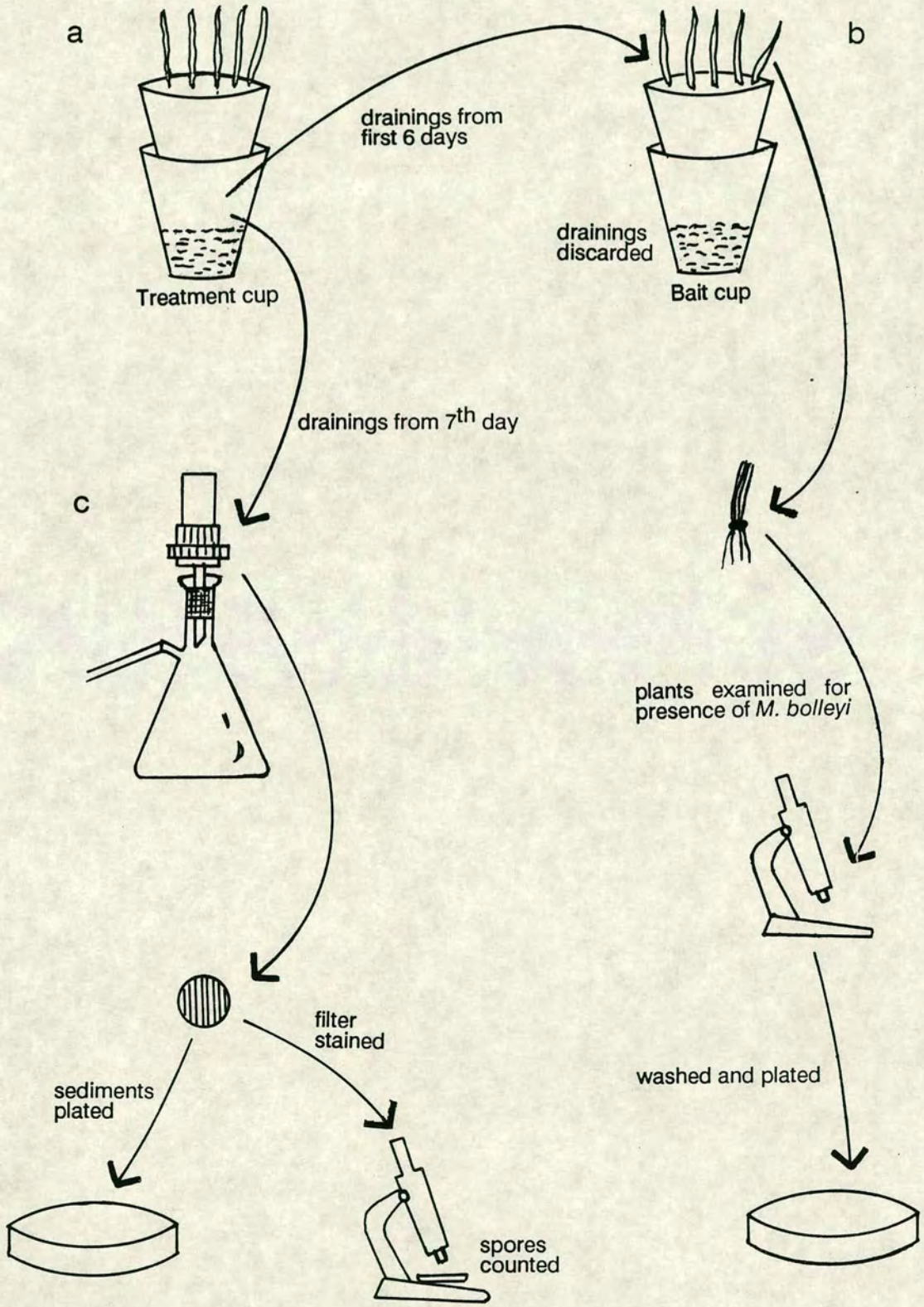
A suspension of spores of *M. bolleyi* (strain T560R1, tolerant of benzimidazole fungicides) in 1% sodium alginate was prepared by adding 10-20 ml of autoclaved 1% sodium alginate solution to 8 day old cultures of *M. bolleyi* on PDA in Petri dishes. The surface of the culture was scraped gently with a sterile glass rod, the spore suspension was passed through a double layer of sterile lens cleaning tissue (Whatman, 105) and the spore concentration was adjusted to 3×10^7 spores ml^{-1}, using a haemacytometer. Surface sterilised wheat seeds were immersed in the spore suspension, then removed one by one with forceps and dropped in autoclaved 0.25% $CaCl_2$ solution. A thin coat of calcium alginate with spores of *M. bolleyi* entrapped in it was thereby formed around the seed (estimated as 0.035 ml $seed^{-1}$ and containing about 10^6 spores per seed). Then the coated seeds were removed, washed three times in distilled water, drained on sterile blotting paper and coated again with two more layers of sterile (spore-free) calcium alginate, to ensure entrapped of the spores on the seed.

For comparison, some wheat seeds were coated as described above with three layers of calcium alginate but without spores. Additionally, spore-containing but seed-free pellets of calcium alginate were made by dropping small drops (0.03 ml) of autoclaved 1% sodium alginate solution containing spores into 0.25% $CaCl_2$ solution, then the pellets were coated with two more layers of sterile spore-free alginate.

Plastic drinking cups (200 ml) with perforated bases were filled with perlite, then seeds or pellets as described above were placed just below the surface of the perlite, 5 per cup. There were 15 replicate cups for each of the three treatments - spore-coated seed, spore-free seed and spores entrapped in beads. Each cup (termed a "treatment" cup) was suspended over another cup (unperforated) to collect irrigation water (Fig.5.1a). They were enclosed in propagators to avoid excessive evaporation and incubated in a glasshouse at 20±1°C without supplementary illumination. For each treatment cup a corresponding "bait cup" was prepared, containing perlite and five surface-sterilised (uninoculated) wheat seeds (Fig.5.1b). The bait cups were raised on inverted cups to avoid contamination between them.

The treatment cups were irrigated on alternate days with 50 ml Hewitt's mineral solution and 50 ml distilled water. The drainings from each cup were collected and used to irrigate the corresponding bait cup containing uninoculated seedlings. This was done for the first six days of the experiment. On the seventh day the treatment cups were irrigated with 100 ml distiled water and the drainage water was collected and passed through a cellulose acetate membrane filter (Nutflow, Oxoid LTD, 0.45 μm pore size, diam. 47mm) to collect any spores in it (Fig.5.1c).

Figure 5.1. Procedure used to assess spores of *M. bolleyi* that could be washed from roots or seeds of plants in "treatment cups" of perlite. See text for detals.



At this stage a second bait cup was used, and drainings from the waterings of days 8-13 were added to it. At 14 days a 100 ml irrigation was used and drainings were passed through a filter as at 7 days. Then a third bait cup was used for the drainings from 15-20 days and, finally, a 21 day sample was again collected on a filter. In other words, the procedure in Fig.5.1a-c was repeated twice again for drainings from the treatment cups in the second and third weeks of the experiment.

The three filters (7, 14, 21 days) for each treatment cup were processed as follows.

A small amount of the solid material retained on each filter was scraped from part of the filter with a sterile scalpel, placed on agar medium in Petri dishes and spread over the surface with a sterile glass rod. PDA (1/4 strength) amended with 50 µg/ml streptomycin and 50 µg/ml chlortetracycline was used. Each isolation of *M. bolleyi* from the filters was tested for growth in PDA amended with 5 µg/ml of carbendazim to see if the initially used strain was isolated.

The filter was placed on a large slide, stained with cotton blue in lactophenol and examined under a microscope (X250) for the presence of fungal spores. The numbers of spores resembling *M. bolleyi* on the filter were recorded in 10 random microscope fields.

All bait plants were incubated after they had been used to collect washings from the treatment cups. When they were 28 days old they were sampled and assessed for colonization of their roots by *M. bolleyi*. The plants were removed from their cups and the perlite was washed carefully under running tap water. The seed (when present) was left on the plant. The plants were examined under a dissecting microscope against a white background and roots with dark

characteristic bodies of *M. bolleyi* were recorded. The identity of these structures was confirmed by examining them at higher magnification (Figures 5.2, 5.3). Although the seeds and coleoptiles were eventually colonised by *M. bolleyi* and often the characteristic dark bodies were observed on them, this assessment was not taken into account because many other fungi can colonize this senescing tissue and produce black specks, sometimes much like those of *M. bolleyi*.

At the end of the experiment the plants in the treatment cups were also examined for presence of *M. bolleyi*, both by direct microscopical observation and by plating representative lengths of root on carbendazim-containing agar.

In addition to the basic plan of the experiment, described above, seeds were removed from plants in the treatment cups at different times, leaving the plants themselves undisturbed. Seven days after seeding, the seeds were removed from plants of five treatment cups; on the 14th day seeds were removed from plants in a further five cups. After seed removal all treatment cups were irrigated, each with 1 litre of tap water, in an attempt to flush down most of the spores on the plants or in the potting medium, before a further set of bait plants was used to receive subsequent washings.

The experiment lasted three weeks. The roots of treatment plants were then examined for the degree of their colonization by *M. bolleyi*. Two seminal roots from each plant were cut at 10 cm from their base, washed by shaking vigorously in several changes of tap water and finally in sterile distilled water. The roots were cut in 1 cm pieces using aseptic techniques and successive 1 cm segments of one root were plated on selective medium. The successive pieces of

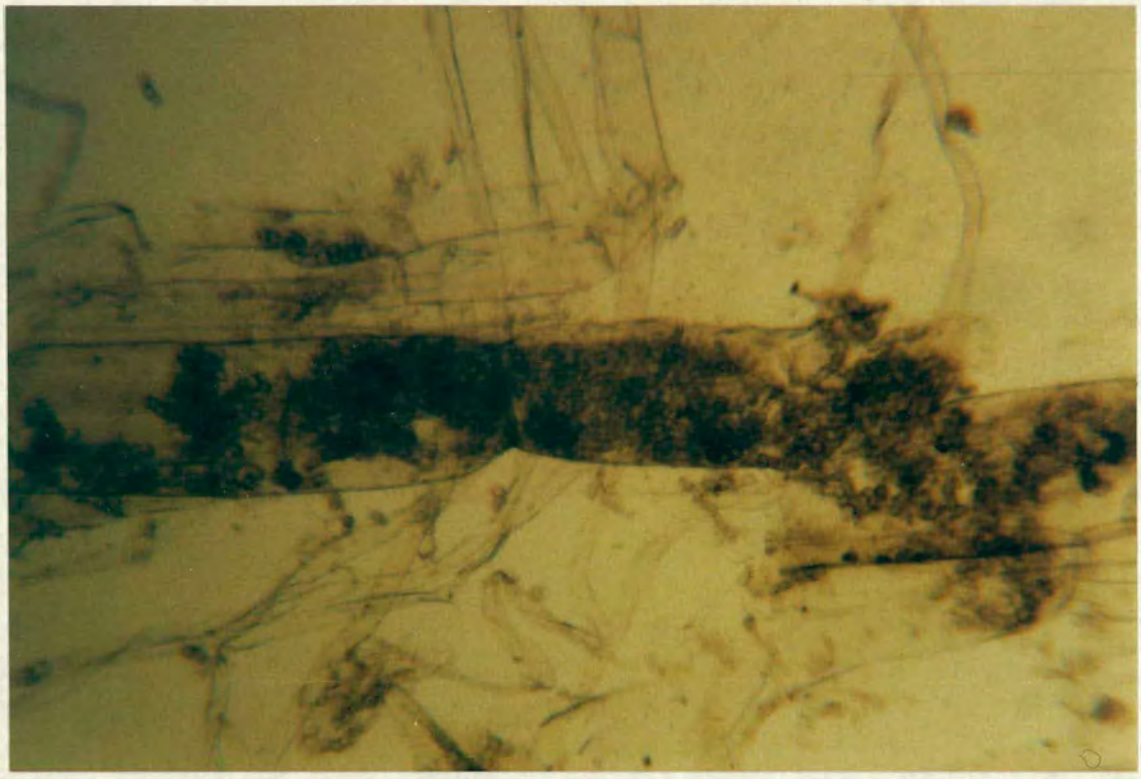


Figure 5.2. Chlamydospore-like structures of *M. bolleyi* in epidermal cells of wheat roots.

Figure 5.3. Clamydospore-like structures of *M. bolleyi* in root hairs of wheat.

the other seminal root were incubated on sterile moist filter paper in Petri dishes for 8 days at 25°C and then examined for the presence of the characteristic dark structures of *M. bolleyi*.

5.2.1. Results

The results of this experiment are shown in Tables 5.1 and 5.2. For convenience they are discussed under different headings below.

Spores recovered on filters

Table 5.1 shows the mean number of *M. bolleyi*-like spores counted on filters (sums of 10 microscope fields per filter) for each treatment. The numbers of spores recovered from cups in which the seedlings were derived from inoculated seeds were much higher than those from cups containing only spore pellets (no seedlings) or non-inoculated seedlings.

Most of the residues on filters from seed-inoculated cups gave rise to colonies of *M. bolleyi* when plated on agar medium, and the colonies were always of the benzimidazole-tolerant strain. In contrast, none of the residues from cups with non-inoculated seedlings yielded colonies of *M. bolleyi*. This assessment confirmed the validity of microscopical assessments of the filters, especially when large numbers of *M. bolleyi*-like spores were observed on filters. There were, however, cases in which a few *M. bolleyi*-like spores had been recorded on the filters from other cups but the fungus was not isolated from these, suggesting that it had been misidentified.

The removal of seeds from inoculated seedlings on the 7th or 14th day resulted in a reduction in the number of spores recovered on filters from these cups at the next sampling. However, the high

Table 5.1. Numbers* of *Microdochium bolleyi*-like spores recovered on filters from washings of cups with inoculated or non-inoculated wheat seeds or with pellets containing spores of the fungus.

	T R E A T M E N T S				
	Inoculated seed				
Day of sampling	Seeds left attached	Seed removed 7th day	Seed removed 14th day	Alginate pellets with spores	Non-inoculated seed
7	69.1 ± 20.6 (15)	n.a.	n.a.	1.4 ± 0.5 (15)	0.1 ± 0.1 (15)
14	165.0 ± 44.0 (10)	45.2 ± 15.9 (5)	n.a.	0.0 ± 0.0 (15)	0.0 ± 0.0 (15)
21	80.4 ± 55.4 (5)	109.6 ± 67.0 (5)	32.2 ± 7.3 (5)	0.5 ± 0.4 (15)	0.2 ± 0.1 (15)

* Mean number of spores detected in 10 microscope fields on each filter with S.E. of means.
In parentheses, number of replicates.
n.a. Not applicable.

variability of spore numbers detected on the filters and the relatively small number of replicates meant that the effects of seed removal on spore production were not statistically significant.

Colonisation of roots of "bait" plants

Table 5.2 shows the percentages of bait plants whose roots had the characteristic chlamydospore-like groups of structures of the fungus. Bait plants receiving drainages from wheat plants inoculated with *M. bolleyi* (and containing high numbers of *M. bolleyi*-like spores as observed on filters) gave high percentages of infected bait plants. There was no correlation between the numbers of bait plants infected with *M. bolleyi* and the numbers of spores observed on the filters. Numbers in parentheses in Table 5.2 show the results of assessments by plating roots of bait plants on selective medium. With this method the fungus was always found to be present on higher numbers of bait plants than those showing visual symptoms.

The removal of the seed early in the life of the plant (7th day) resulted in fewer bait plants showing symptoms, compared to plants with seed attached (P=0.05). But the production of propagules was evidently restored during the third week of incubation of treatment cups. There was no apparent effect when the seed was removed later, at the 14th day, although the numbers of spores on filters in this treatment were reduced as shown in Table 5.1.

Only a small number of bait plants with the characteristic structures of *M. bolleyi* were observed when these plants had received drainage water from cups containing alginate pellets. The numbers of the same bait plants yielding colonies of *M. bolleyi* when plated on selective medium were also low (numbers in parentheses in Table 5.2).

Table 5.2. Percentage of bait plants infected with *Microdochium bolleyi*.

Days of treatment	T R E A T M E N T S				
	Inoculated seed				
	Seeds left attached	Seed removed 7th day	Seed removed 14th day	Alginate pellets with spores	Non-inoculated seed
1-6	23 a* (76) **	n.a.	n.a.	6 (14)	0 (0)
8-13	81 b (100)	25 a (100)	n.a.	0 (10)	0 (0)
15-20	95 b (100)	96 b (100)	91 b (100)	0 (18)	3 (7)

* Figures with letter in common are not significantly different from each other at P=0.05.

** In parentheses, percentage of bait plants infected when roots were plated on selective medium.

Wheat plants in two bait cups receiving drainages from non-inoculated seedlings were colonised by *M. bolleyi*, presumably due to accidental contamination.

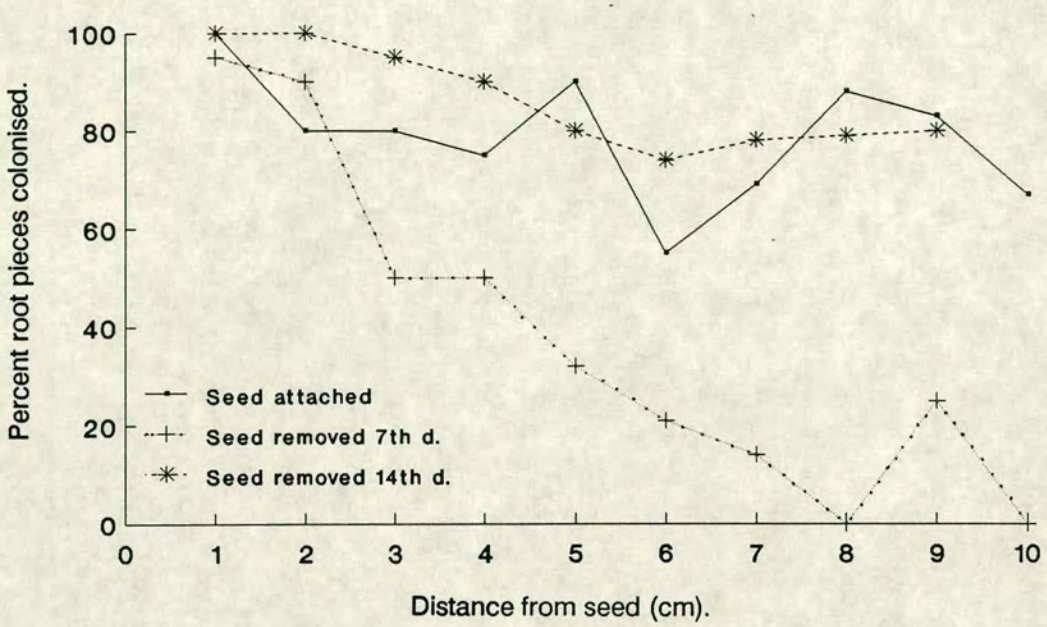
Colonisation of roots of "treatment" plants

M. bolleyi was isolated from the whole upper 10 cm length of roots of seed-inoculated plants when the roots were plated on selective medium, irrespective of seed removal. There were differences, however, in the presence of the characteristic structures of the fungus along the root length among the different treatments involving seed removal when the roots were cut in 1 cm pieces and incubated for a 8 further days on filter paper. In plants whose seed was removed on the 7th day the percentage of root length having these structures was 47%, significantly less ($P<0.001$) than in the two other treatments (no-removal or seed removal on the 14th day -79% and 86% respectively). Figure 5.4 shows the distribution of these characteristic structures of *M. bolleyi* along roots of plants in these three treatments. Late seed removal and "no-removal" resulted in a higher and more uniform distribution along the roots, while "early seed removal" resulted in these structures being present mainly in the upper part of the roots. *M. bolleyi* was not detected in roots of non-inoculated plants.

5.3 Experiment 2: colonisation of roots by *M. bolleyi* from inoculum applied as beads.

A similar experiment to that above was done using "bait plants" and detection of spores on filters, but the experimental details differed as follows.

Figure 5.4. Per cent of successive 1 cm root pieces bearing chlamydospore-like structures of *M. bolleyi*, when the seed was present or was removed from inoculated plants at different times.



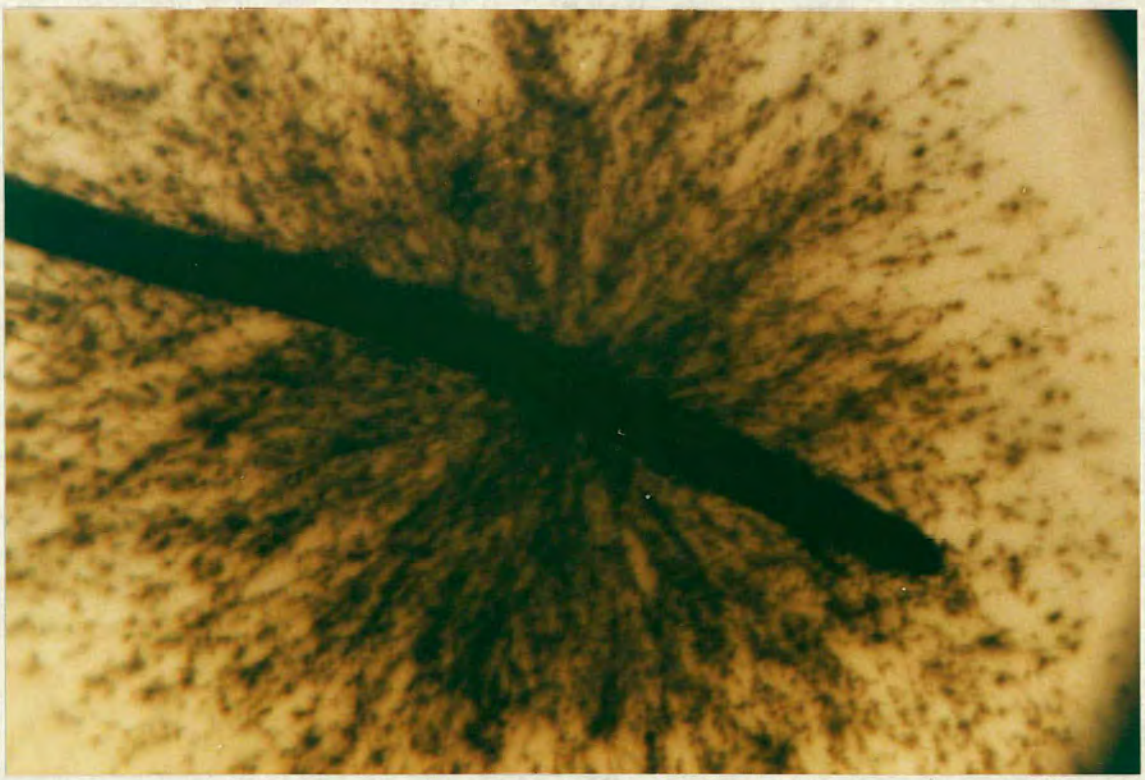


Figure. 5.5. Typical colony of *M. bolleyi*, growing from root plated on selective medium. The chlamydospore-like structures of the fungus appear in the agar after incubation for 5-6 days at 25 ºC.

Wheat and tomato (cv ACE 55) were included in the experiment-the latter plant being considered as a non-host of *M. bolleyi*. The seeds were not coated with alginate nor were they inoculated directly. Instead the inoculum was placed 1cm below the germinating seeds and it consisted of spores of *M. bolleyi* entrapped in calcium alginate pellets. These were made from 0.03 ml sodium alginate solution containing spores ($2X10^7$ ml^{-1}) and they were coated twice with spore-free calcium alginate as described earlier. Sterile calcium alginate pellets were included as controls. Plastic cups were filled with perlite up to a fixed level, then five calcium alginate pellets were placed in marked sites, a 1 cm layer of perlite was added over the pellets and five surface-sterilised seeds of wheat or tomato were placed over the positions of the pellets. The seeds were covered with a further 1 cm layer of perlite. The following five treatments were included.

(I) Wheat seeds above pellets with spores of *M. bolleyi*.

(II) Wheat seeds above control pellets (no spores).

(III) Tomato seeds above pellets with spores of *M. bolleyi*.

(IV) Tomato seeds above control pellets (no spores).

(V) Pellets with spores of *M. bolleyi*, but no seeds.

There was also a sub-treatment of seed removal in 7 (out of 15) of the cups containing wheat plants when they were 18 days old.

Drainings from the cups were applied to bait plants and collected on filters (as in Fig. 5.1) for five successive weeks, with weekly changes of bait plants and filters. Each set of bait plants was assessed for infection by *M. bolleyi* 4 weeks later. Although this is usually enough time for *M. bolleyi* to form its characteristic structures on roots, few of the bait plants bore these structures, so root colonisation was further assessed by

washing and plating the top 1 cm of each seminal root on selective medium (Fig. 5.5).

Seven of the "treatment cups" of wheat and tomato were sampled when they were 6 weeks old and the rest when they were 8 weeks old. At sampling, the root lengths were washed and plated on selective medium and the regions from which the fungus had emerged after 3 days were recorded. From the wheat plants all seminal roots (excluding the laterals) were plated after they had been severed 10 cm from their base. From tomato plants, the tap root and five well developed laterals up to 10 cm long were taken at random and plated on the selective medium.

5.3.1. Results

Detection of *M. bolleyi* on bait plants or filters

The results from this experiment are shown in Table 5.3. In contrast with the previous experiment, only few *M. bolleyi*-like spores were detected on filters from cups containing inoculum of *M. bolleyi* during the 5 weeks of this experiment (numbers in parentheses in Table 5.3). The low numbers of spores is also reflected in the very few cases of isolation of *M. bolleyi* from the sediments on the filters. It is notable however that *M. bolleyi*-like spores (albeit in very low numbers) were detected most frequently from cups containing inocula of *M. bolleyi* together with plants. The small numbers do not permit statistical analysis.

Only 6 out of 347 bait plants in the treatment "wheat + inoculum" had the characteristic structures of *M. bolleyi* on their roots although the fungus was found on 59 of them when assessed by plating root segments on selective medium, and these latter results are used to obtain the percentages shown in Table 5.3. Again the

Table 5.3. Percentage* of bait plants whose roots yielded *Microdochium bolleyi* when plated on selective medium, and numbers of *Microdochium bolleyi*-like spores (in parentheses) recovered on filters at corresponding times.**

Days of treatment (or sampling of spores on filters)	T E A T — Wheat + inoculum: Seedlings with seed attached	Wheat + inoculum: Seedlings with seed removed at 18 days	M E N T S: Tomato + inoculum	Inoculum alone (pellets)	Wheat with no inoculum (control)	Tomato with no inoculum (control)
1-6 (7th)	1.8±1.8 (9)	n.a.	0 (3)	5.5±3.0 (2)	0 (0)	0 (2)
8-13 (14th)	19.6±9.0 (0)	n.a.	19.4±8.4 (2)	6.0±4.5 (2)	0 (0)	0 (0)
15-20 (21st)	11.3±11.3 (0)	9.4±6.2 (1)	0 (0)	0 (0)	0 (0)	0 (0)
22-27 (28th)	31.2±13.9 (6)	0 (0)	0 (3)	0 (0)	0 (0)	0 (0)
29-34 (35th)	38.7±13.7 (5)	23.8±7.7 (0)	0 (0)	4.2±6.0 (0)	0 (0)	0 (0)

* Percentages, transformed to arcsine, with SE.

** Mean number of spores detected in 10 microscope fields on each filter.

high variability of the results does not permit statistical analysis and comparisons between the treatments. It is notable however that drainings from wheat plants resulted in increased numbers of infected bait plants compared with those from tomato plants; fewer bait plants were infected from cups containing the inocula alone (pellets) and none from the non-inoculated wheat and tomato treatments (controls).

It is clear from Table 5.3 that the assessment of bait plants is a more sensitive method of detection of the fungus than is assessment of spores on filters, for an estimate of propagule production from treatment cups. Notable also is that propagule production from wheat plants (assessed by baiting) was relatively constant over time after the 8th day, whereas propagule production from cups containing tomato plants was detected only in the second week of the experiment.

Removal of wheat seeds on the 18th day resulted in an apparent reduction in the production of propagules detectable by baiting or on filters. This difference was not statistically significant but it was consistent in all three later samplings. None of the removed seeds was found to be colonized by *M. bolleyi* when plated on selective medium.

Colonization of roots of treatment plants

M. bolleyi was isolated from roots of 6- and 8-week old wheat and tomato treatment plants. Its presence ranged from 0 to 100% of the root lengths assessed for wheat plants and from 0 to 57% in roots of tomato plants. The highest percentages concern only one plant in one cup for each species. The rest of the plants had lower percentage of colonisation of their roots and, excluding the two

cups with exceptionally high degrees of root colonisation, the wheat treatment plants had a mean of 15% of their root length colonised while for tomato roots this was only 2%, localised at the place of the inoculum.

The percentage of root length colonised for all wheat plants pooled from the two sampling dates was 16.7%. Plants sampled when 8 weeks old had a somewhat higher percentage of colonisation (18.8%) compared to those sampled when 6 weeks old (14.4%), although this was not statistically significant. Roots of tomato plants yielded fewer colonies of *M. bolleyi*, mainly from laterals in the vicinity of the inoculum. There was also a slight increase in the percentage of tomato root length colonised by the fungus with time, from 1.2% in those plants sampled the 6th week to 2.7% in those sampled the 8th week (9.5% if the cups with exceptionally high degrees of colonisation are included).

There was a difference in root colonisation between plants whose seed was removed when they were 18 days old and those with seeds *in situ* (12.4% and 20.6% respectively, pooled for all sampling dates; significant at P=0.05), reflecting the differences found in the bait plants and in spores on filters. None of the seeds removed from these plants on the 18th day was found to be colonized by the fungus but seeds from 8 of the 30 plants with seed attached were found at the end of the experiment to be colonized by *M. bolleyi*. There was a significant correlation (p=0.05) between the percentage of root colonization of wheat plants in each treatment cup and the percentage of plants in the corresponding bait cups colonized by the fungus, in the experiment as a whole.

Those plants whose seed was colonized by *M. bolleyi* had a higher percentage of root length colonization (53.9%) compared to those whose seed was not colonised (13.7%).

Figure 5.6. Typical patterns of colonisation of roots of "treatment" plants by *M. bolleyi* (experiment **2.**). The first row shows five wheat plants from one cup that were colonised to a low degree, and the second row shows wheat plants from a cup colonised to a higher degree. The third row shows typical patterns of root colonisation in two tomato plants. Numerical values are percentage of the plated root system for which *M. bolleyi* was isolated.

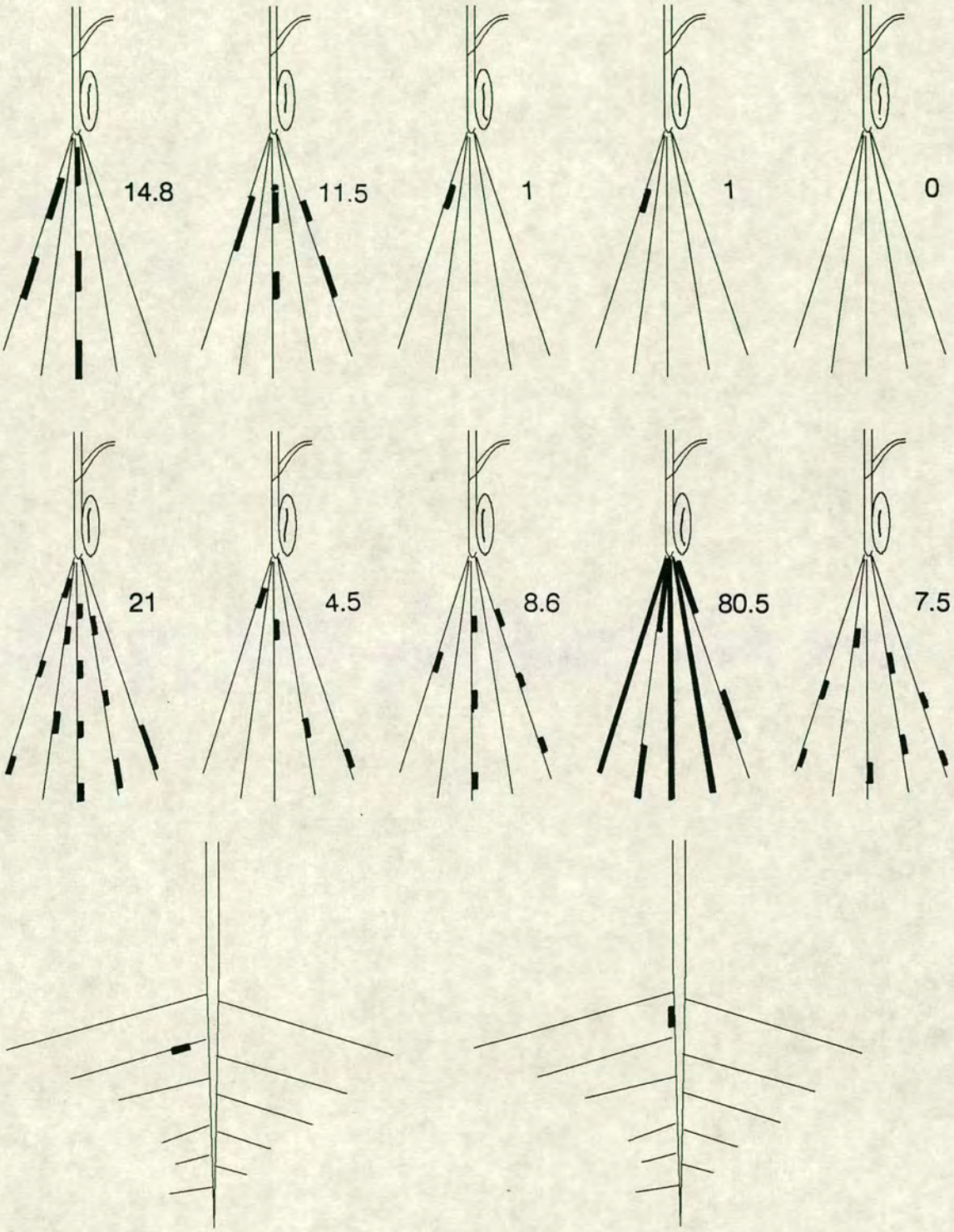


Figure 5.6 shows some typical patterns of root colonisation of wheat and tomato in treatment cups in this experiment. The interesting features revealed by this figure are, first, that colonisation was discontinuous on individual roots and, second, that some individual roots were heavily colonised whereas others were apparently uninfected.

5.4. Experiment 3: production of spores on seeds and roots

In order to estimate the numbers of spores produced on seeds and roots of wheat seedlings and the effect of seed removal, a different and more direct approach than in the previous experiments was used. Wheat seedlings derived from seed inoculated with alginate as in experiment 1 were grown in perlite in plastic cups in propagators, 5 seeds per cup and 4 replicate cups per treatment. The cups were irrigated only from their base by capillarity, to avoid any washing of spores down the profile. The treatments involved seed removal after 7 and 14 days as in experiment 1 together with no seed removal.

When they were 1, 2 and 3 weeks old, plants were removed gently from the potting medium leaving aggregates of perlite on the roots. The first seminal root was removed and kept in 70% methylated spirits for assessment of RCD, then the other roots and the seed (if present) were removed and placed in small bottles with sterile distilled water. The seeds or roots were shaken vigorously, serial dilutions were made and 0.5 ml aliquots of these were spread on agar medium in Petri dishes. The medium was quarter-strength PDA amended with streptomycin 50 μg/ml, chlortetracycline 50 μg/ml and carbendazim 5 μg/ml. The number of colonies that developed in 48h was counted. The dishes were kept a few more days to ascertain that

all colonies were of *M. bolleyi*. The same roots used for assessment of spore numbers were also examined for their extent of colonisation by *M. bolleyi*. For this, they were washed several times by shaking vigorously in tap water and finally in sterile distiled water. Then half of the roots of each plant were surface sterilized by shaking for 30 sec in saturated calcium hypochlorite solution and all (including those not surface sterilized) were cut into successive 1 cm pieces and plated on water-agar amended with streptomycin 50 μg ml^{-1}, chlortetracycline 50 μg ml^{-1} and carbendazim 5 μg ml^{-1}. The root segments that gave rise to colonies of *M. bolleyi* were recorded after 5 days.

5.4.1. Results

Table 5.4 shows the numbers of spores found on seeds and roots. During the first 3 weeks the number of spores produced on the seeds increased progressively. This increase was significant (P=0.02) for the number of spores on seeds in the third week compared with the two previous samplings. The number of spores obtained from roots was relatively small in the first week, but increased in the next two weeks, even when seeds were removed. The most important point revealed by this is the great contribution of the seed to spore production during the germination and the early period of the seedling's life. This pattern of spore production is in accordance with the findings in experiment 1 where sporulation was assessed indirectly, using filters and bait plants.

Table 5.5 shows the percentage colonisation of the first 10 cm length of seminal roots, assessed with or without surface sterilisation. Even by the 7th day a substantial proportion of root length was infected and at least some of this colonisation was

probably deep enough in the cortex such that the fungus was unaffected by surface sterilisation. It is notable that the degree of colonisation increased to near maximum between the 7 and 14 day samplings, and that removal of seed on the 7th day had no marked effect on the degree of root colonisation by *M. bolleyi*. There was a low (r=0.62) but significant (P=0.005) correlation between the percentage of root colonization of each plant and the numbers of spores found on their roots.

Table 5.4. Numbers (X 10^3) of spores of *M. bolleyi*, recovered by dilution plating from seeds and roots of wheat seedlings derived from seeds inoculated with spores of the fungus; in parentheses, numbers of spores per cm root length.

PART OF THE PLANT AND TREATMENT	DAY OF SAMPLING 7	14	21
Seed	168 ± 46	229 ± 45	457 ± 54
Roots (Seed attached)	22 ± 06 (1.7)	146 ± 58 (5.5)	105 ± 25 (3.8)
Roots (Seed removed the 7th day)	n.a.	194 ± 126 (9.2)	122 ± 42 (7.5)
Roots (Seed removed the 14th day)	n.a.	n.a.	91 ± 33 (3.0)

Means and S.E. of 4 replicate cups.

Fig. 5.7 shows, for pooled results of all plants at the 21 day sampling, the pattern of reisolation of *M. bolleyi* from successive distances along surface sterilized roots and the pattern of RCD in the first 10 cm of the first seminal roots. Cortical senescence was almost negligible at the 7 day sampling (results not shown) but increased progressively with plant age. By 21 days the epidermal layer was dead along the 10 cm length of almost all roots and senescence had advanced to the inner cortical layers in the basal portion of the roots. There was a significant correlation between the amount of RCD and the occurrence of the fungus in successive 1 cm lengths of the roots (r=0.64, P =0.001).

Table 5.5. Percentages* of root length colonised by *M. bolleyi*, on wheat seedlings derived from seed inoculated with spores of the fungus, with seed attached or removed on the 7th or 14th day.

TREATMENT	DAY OF SAMPLING		
	7	14	21
Seed attached	52.2±3.2 (30.2±4.1)	81.5±5.1 (51.1±3.4)	89.0±1.0 (43.9±4.8)
Seed removed the 7th day.	n.a.	90.0±0.0 (67.5±8.8)	90.0±0.0 (43.3±11.3)
Seed removed the 14th day	n.a.	n.a.	90.0±0.0 (45.3±7.7)

* Percentages and S.E. of data transformed to arcsine; means of 4 replicate cups. In parentheses % root length that yielded *M. bolleyi* after surface sterilisation.

Figure 5.7. Mean number of nucleate cortical cell layers (first seminal root axis) and percentage of other seminal roots that yielded *M. bolleyi* at different distances from the seed after being surface sterilised and plated on selective medium. Data from Experiment 3 pooled for all treatments of seed removal.

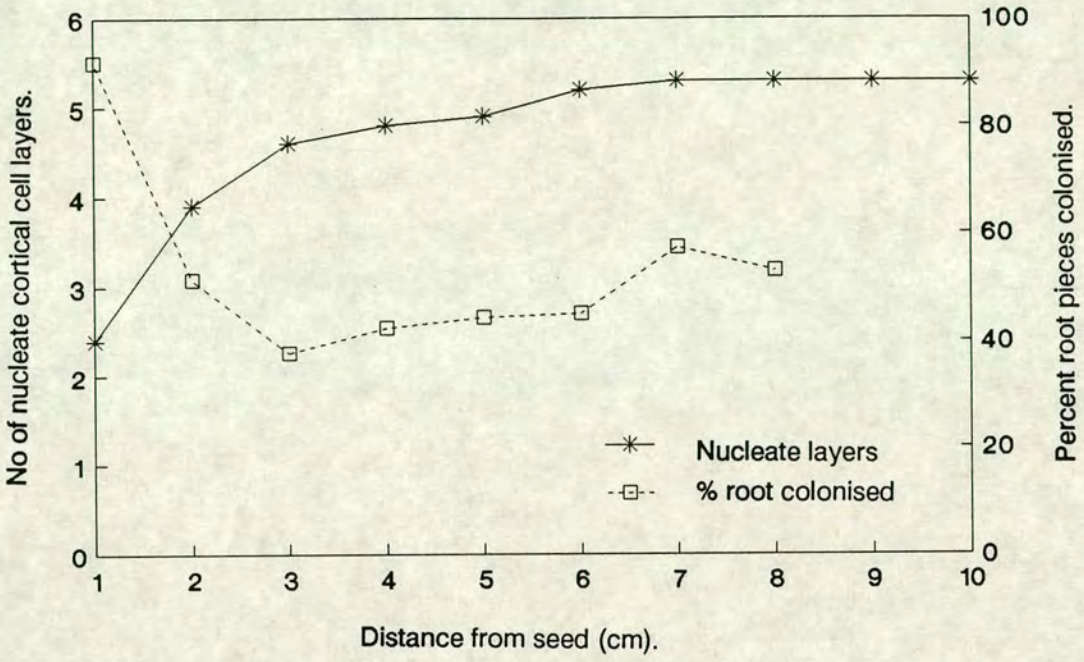
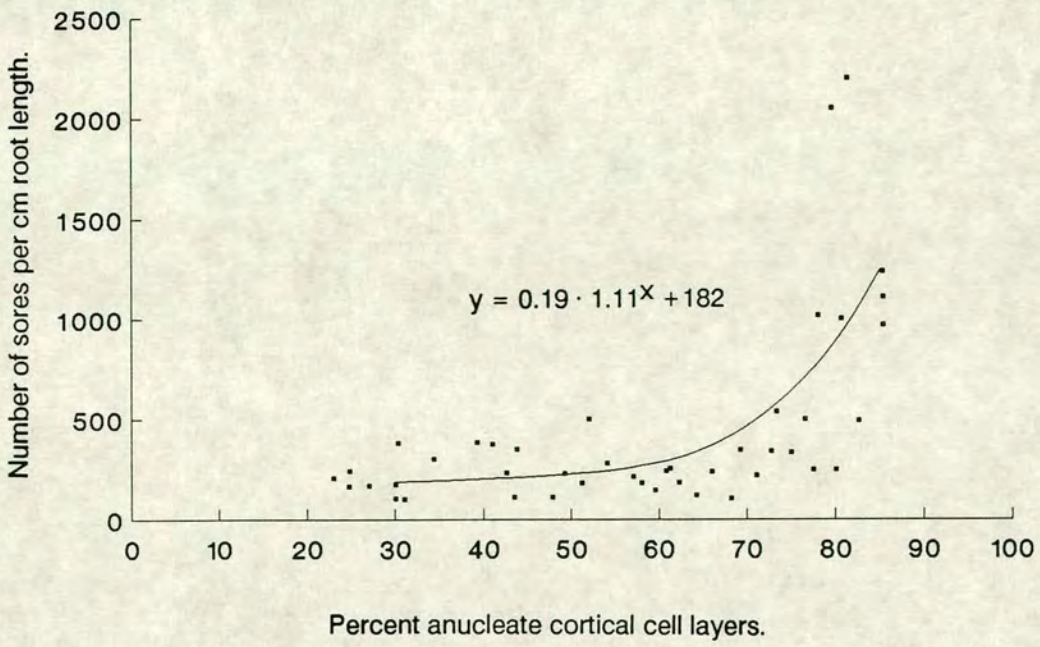


Figure 5.8. Relationship between percentage of anucleate cortical cells in wheat seminal roots and number of spores of *M. bolleyi* recovered per cm of the same roots of plants inoculated when 10 days old and sampled when 30 days old.



5.5. Experiment 4: sporulation from seeds in the absence of calcium alginate, and assessment of the efficiency of washing of spores through perlite

In this experiment, wheat seeds were surface sterilized as in experiment 1 and were then treated in two ways. Spores of *M. bolleyi* strain T560R1 were applied in calcium alginate as in experiment 1 or the seeds were dipped in an aqueous suspension of $2X10^7$ spores per ml then sown immediately in plastic cups with perlite. There were five seeds per cup and eight replicate cups per treatment. Each alginate-coated seed received about 10^6 spores, while each seed treated with the water suspension had about 10^5 spores. A set of four cups was also included as a supplementary part of the experiment, to assess the efficiency of washing spores from the cups with irrigation water.

The cups were placed in the glasshouse in the same conditions as in experiment 1, but were not irrigated from the top in order to avoid any disturbance and dispersal of the spores produced on the surface of the seeds. Instead they were placed in shallow trays and were irrigated by capillarity from their base with half-strength Hewitt's nutrient solution.

At sampling, the perlite was removed gently from the top of the cups and the seeds were removed carefully using sterile forceps. They were placed in 20 ml vials containing 10 ml sterile distiled water, shaken vigorously and serial dilutions were made, samples of 0.5 ml being spread on agar medium selective for *M. bolleyi* as described earlier.

For the assessment of washing of spores from the plants, at 7 and 14 days after seeding the cups were flushed twice with 1 litre of tap water each time. The draining water was collected separately

Table 5.6. Number* of spores produced on wheat seed during germination and early development of the plant, when the inoculum was applied as a suspension of spores in water or entrapped in alginate gel.

Age of plants in days	Spores per seed. ($X10^3$)	
	Alginate coated	Spores applied in water
7	127 ± 10	266 ± 31
14	253 ± 44	611 ± 182
21	375 ± 28	965 ± 280

* Means ± SE for 4 replicate cups.

Table 5.7. Numbers of spores ($X\ 10^3$) recovered in two successive irrigations, each with 1 l water, from cups with 7 and 14 day old wheat seedlings.*

Day	First irrigation	Second irrigation
7	710 ± 220	160 ± 30
14	1240 ± 550	270 ± 110

* Means ± SE for 4 replicate cups.

and the numbers of spores in it were assessed by plating serial dilutions onto selective medium.

5.5.1. Results

Table 5.6 shows the number of spores produced on seeds of wheat plants when these were inoculated with a spore suspension in water or coated with calcium alginate. It is clear from the results that calcium alginate coating repressed or delayed sporulation by the fungus, the numbers of spores in the calcium alginate treatment being consistently lower than when spores were applied in water. Although the numbers of spores found on seeds coated with calcium alginate were much lower than with the same treatment in the previous experiment, the spores recovered from treatments without calcium alginate greatly exceeded the numbers from alginate-coated seeds in this and the previous experiment.

Table 5.7 shows the numbers of spores retrieved from cups in two successive washings at both the 7th and 14th day of incubation. The second washing gave spore numbers about a quarter or a fifth of those in the first washing. These results suggest that even with the large amount of the water used in this experiment, the washing of spores from the cups was not complete.

5.6 Experiment 5: spore production from roots of different age and with different amounts of RCD

In this experiment surface-sterilised wheat seeds were placed, one per container, in 50 cm long polyethylene tubes of 5 cm diam. filled with perlite. The tubes were sloped so that the roots were growing against the transparent polyethylene surface. The roots were kept in darkness by inserting the transparent tubes in rigid opaque

plastic tubes of slightly larger diameter, but were removed carefully every day so that growth of the root could be recorded by marking the positions of the root tips on the polyethylene. Ten days from seeding, the roots were inoculated by irrigating each tube with 500 ml water suspension of spores of *M. bolleyi*, 10^4 spores per ml. The tubes were left for 7 days in order for the fungus to be well established on the roots, then on the three following days (days 8, 9 and 10) all tubes were flushed with one litre of tap water in an attempt to remove as many spores as possible from the roots and the rooting medium. Nine plants were sampled 10 days later. For this, the polyethylene tube was cut and root segments were taken from the marked roots, corresponding to regions aged 0-4, 5-7, 8-10, 11-14, 15-20 days. The numbers of spores present on these root regions were assessed by shaking the roots in distilled water and making serial dilutions for plating on selective medium. The total root length and cortical senescence was assessed along the whole root length after staining with AO. Tubes that were not sampled 10 days after inoculation were turned through 180° along their axes and irrigated carefully so that the water did not pass along the roots (which were now against the uppermost surface of the polyethylene tubes). These tubes were left for 10 more days and then sampled. Spore numbers recoverable from root regions, root length and RCD were assessed as before.

5.6.1. Results

Table 5.8 shows results of number of spores recovered from wheat roots at the first and second samplings. From the relatively high numbers of spores found on roots at the first sampling it seems that the preceding three daily flushings were not effective in removing

Table 5.8. Numbers* of *M. bolleyi* spores on roots in relation to root age and degree of cortical senescence, when wheat seedlings were inoculated when they were 10 days old and sampled at 20 and 30 days.

20-day plants			30-day plants		
Age (days) of root segments at sampling	% root cortex dead**	No of spores cm^{-1} root length	Age (days) of root segments at sampling	% root cortex dead**	No of spores cm^{-1} root length.
15-20	40.0±1.6	209±49	25-30	63.8±1.4	1145±213
11-14	26.6±1.2	143±22	21-24	56.5±1.8	356±42
8-10	25.8±1.5	173±19	18-20	48.3±1.6	195±28
5-7	14.8±1.4	187±33	15-17	42.2±2.1	236±34
0-4	0.0±0.0	101±27	10-14	34.1±2.8	300±113

* Means ± S.E. for 9 replicate tubes.

** Arcsine-transformed.

spores from the roots. Nevertheless, consistently higher numbers of spores were found on roots in equivalent regions 9 days later, showing that spores continued to be produced on roots of all ages and especially in the older root regions.

Figure 5.8 shows the relationship between the numbers of spores detected per centimetre of root length and the amount of RCD of the same root regions. Attempts to fit various curves to the data were not entirely satisfactory, but a plot of an exponential function is shown in Fig.5.8 for data from the 30 day sampling. The relevant equation is $y = 182 + 0.192 \cdot 1.1067^{x}$, where y is the number of spores and x is percentage RCD. The most notable feature of the data points is that there was little or no difference in recoverable spore numbers from regions of roots showing between 20% and 70% death of cortical cell layers, but there was a steep rise in spore

Table 5.9 Presence of chlamydospore-like structures of *M. bolleyi* on wheat root regions of different ages on 30-day old plants (experiment 5).

Age of roots in days	Chlamydospore-like groups cm^{-1} root length*
25-30	5.75±1.40
21-24	0.56±0.02
18-20	0
15-17	0
10-14	0

* Means ± S.E. for 9 replicate tubes.

numbers recoverable from root regions with 80% or more of the cortex anucleate. The way this experiment was done precluded any artefact caused by failure to wash the spores down the lengths of the tubes, as seen by the uniform distribution of spores along roots at the first sampling (Table 5.8).

As shown in Table 5.9, chlamydospore-like bodies of *M. bolleyi* occurred only or mainly in the oldest regions of roots, which were generally those exhibiting most RCD. However, the relevance of this association may be questioned because chlamydospore-like bodies were not seen in other regions of the root, further from the seeds, even when such regions had amounts of RCD equivalent to those in the oldest regions.

5.7. Experiment 6: susceptibility to infection and subsequent sporulation on roots of plants of different age

In this experiment the relationship between age of root regions and their susceptibility to infection and ability subsequently to produce spores was investigated. The experiment was done in 35 cm long polyethylene tube filled with perlite and held in a vertical position. Each tube received two wheat seeds that had been pre-germinated for 24 h and the tubes were irrigated with 200 ml mineral nutrient solution and 200 ml distilled water on alternate days. There were five sets of tubes, seeded at about weekly intervals, 12 tubes for each seeding time. The roots of the seedlings were observed at 7 and 14 days after sowing, when the positions of the root tips against the polyethylene walls of the tubes were marked.

When the plants were 27, 20, 11, 3 and 0 days old, eight tubes of each age set were inoculated with *M. bolleyi* strain T560R1, the

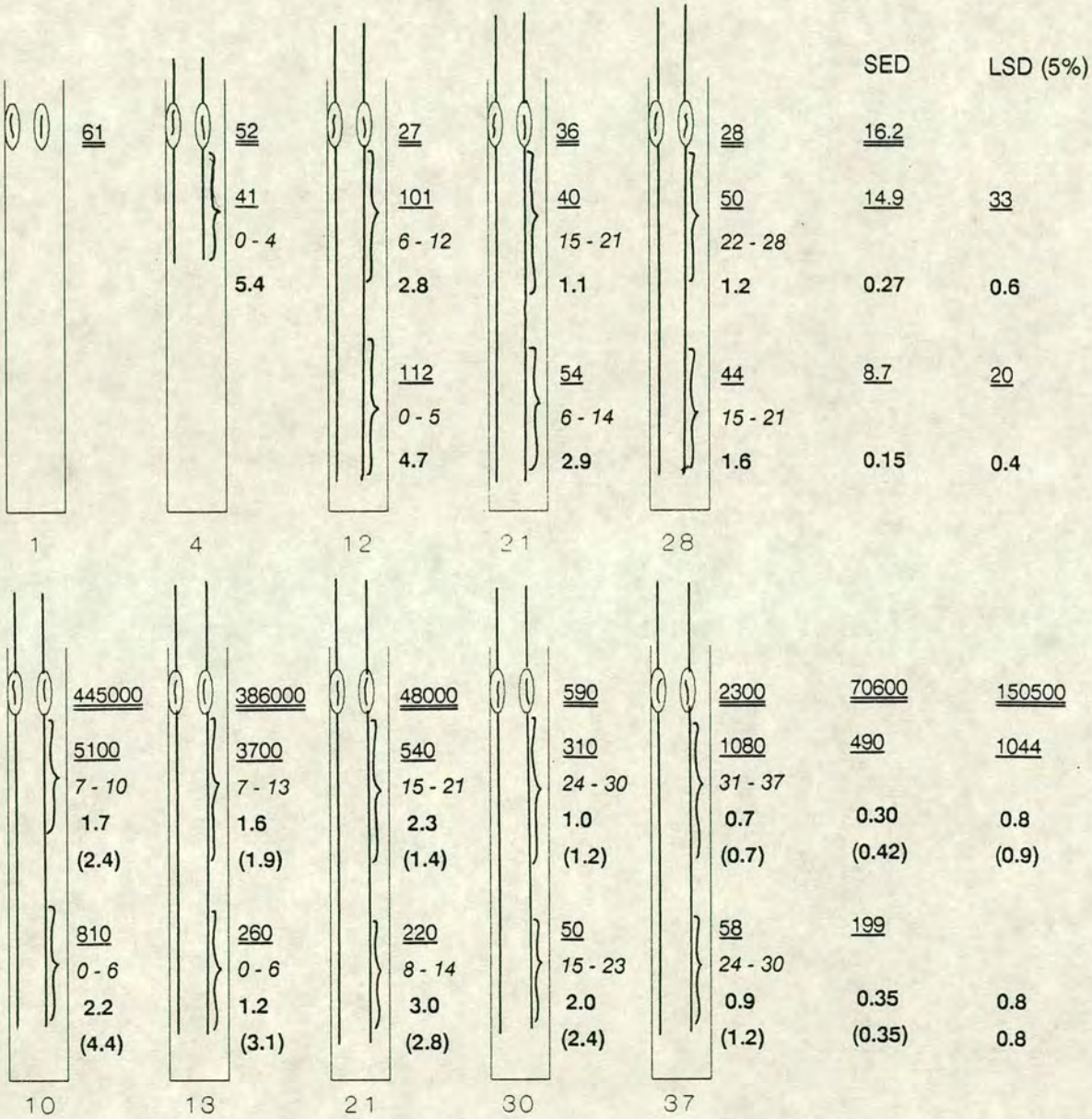
remaining four tubes of each set being left uninoculated as controls. Inoculation was done by irrigating each tube with 500 ml of a suspension containing 10^4 spores/ml in Hewitt's mineral nutrient solution. Twenty four hours after inoculation, four inoculated tubes of each age group were sampled. The rest -inoculated and controls- were sampled 10 days after inoculation. At sampling, each polyethylene tube was cut along its length and root pieces of known age (marked) were removed carefully; the perlite was then removed gently from these roots and the laterals also were removed. The numbers of *M. bolleyi* spores on seeds and root segments were assessed using serial dilution and plating on selective medium, as described previously. RCD of the sampled root pieces was assessed in representative root pieces 15 mm long (at least 8 pieces per sample); this was done by first cutting a root in half and then taking the 1.5 cm pieces for assessment of RCD from midway along the older half and the younger half of the roots.

5.7.1. Results

The results are presented in Figure 5.9. The top series of diagrams represents tubes with seedlings of different ages sampled 24 h after inoculation. There were no controls (non-inoculated) in this sampling, because it was not expected that spore inoculum would have any effect on plant-derived spore numbers or RCD at this time.

The numbers of spores recovered from seeds (double-underlined) and roots (underlined) 24 h after inoculation were very low and almost certainly were spores that were applied at inoculation and not produced in this short interval. In contrast, the number of spores per seed and per cm root length 9 days later (at the second sampling; bottom row of diagrams in Fig.5.9) were much higher and

Figure 5.9. Schematic representation of the design and results of Experiment 6. Each treatment, is shown in the figure as a "tube". Numbers at the bottom of each tube show the age of plants (days) at sampling. The upper row of tubes presents the first sampling, 24 h after inoculation, and the second row the results of equivalent tubes at the second sampling, 10 days after inoculation.



Numbers to the right of each tube show results of assessments as follows. Mean number of spores per seed, double underlined; mean number of spores per cm of root length, underlined; age (days) of the relevant part of the root, italised; mean number of nucleate cortical cell layers, bold; mean number of nucleate cortical cell layers of uninoculated plants (second row only), bold in parentheses. All means and SED are from 4 replicate tubes per treatment.

reveal a strong relationship between spore production and age of the plants at inoculation. Most spores were recovered from seeds of plants inoculated 0 or 3 days after sowing. Seeds of plants inoculated 11 days after sowing produced fewer spores and these inoculated 20 and 27 days after sowing produced even fewer.

The numbers of spores found on roots were also related to plant age at the time of inoculation, the higher numbers being found on roots of plants that were very young at inoculation. This was true for both the older and younger regions of the root systems, although the latter always yielded fewer spores than did the older root regions within an age set of tubes. Such differences were not related to the amount of RCD or to the age of root regions in the experiment as a whole, and also were not related to the amount of RCD in sampled root regions within the age sets of plants.

Young inoculated roots had increased RCD compared to that in uninoculated controls, the difference sometimes being as much as 2 cell layers, but there were no such differences in roots of the older plants. Two-way analysis of the amount of RCD among the different groups of roots and the inoculation treatments showed a significant (P=0.01) increase of RCD in the inoculated, compared to non-inoculated plants.

Overall, there was a large amount of RCD in this experiment, probably due to high temperatures in the glasshouse, reaching 30°C during the hotter days.

5.8. Discussion

Experiments in this section showed that seed treatment is a very effective way to introduce *M. bolleyi* into the wheat rhizosphere. Much less effective colonisation of wheat roots was achieved by introducing the fungus in alginate pellets below the germinating

seeds. Then the roots of inoculated plants did not show visual symptoms (chlamydospore-like groupings) and establishment of the fungus was highly variable along the roots. Part of the reason for this difference may have been that roots encountered the inoculum only by chance if it was applied below the seed. In this respect the different ability of the fungus to establish on wheat compared with tomato roots could have been due to a difference in rate and density of root growth of these plants, quite apart from inherent differences in their abilities to support growth by the fungus. But a major factor that influenced the greater ability of *M. bolleyi* to spread from seed-applied inocula rather than from alginate pellets beneath the seed may have been the food resources available to the fungus close to its inoculum.

Increased efficiency of biocontrol agents is often reported when they were incorporated with a food base into soil (Chet *et al.*, 1979; Backman & Rodriguez-Kabana, 1975). In this respect introduction of *M. bolleyi* by seed inoculation enables the fungus to utilise seed resources and gives it priority on this food base.

Spores were found to be produced abundantly on germinating seeds and roots of wheat seedlings derived from seed inoculation with *M. bolleyi*. Fewer spores were produced from roots encountering an inoculum placed below the seed, as evidenced by the use of bait plants and filtering methods to collect spores that were drained from plant containers.

Several aspects of the results in this section demonstrated both the efficiency of spore production by *M. bolleyi* on plants and the ability of the spores so produced to be distributed in percolating water and to initiate infection of further plants -a model of their abilities to spread down the root system of a single plant. For

example spores were produced and washed down for at least 5 weeks after seeding. They were produced on plants with attached seed or those with seeds removed when plants were 7 or 14 days old. Seed removal resulted in decreased spore recovery in the first experiment, suggesting that a considerable proportion of the spores are produced on the seed. This was confirmed in the following experiments where a more direct estimate of numbers of spores was made. Newly formed spores were observed microscopically on seed coats as early as 48h after inoculation and seeding. So it seems that sporulation on the seeds in the conditions of these experiments was an important early event leading to spread of *M. bolleyi* down the root systems. The conditions were, of course, artificial. In particular, it is possible that the alginate coating on seeds precluded the early establishment of other organisms on the seed coats and thus could have given *M. bolleyi* an early competitive advantage. Alginate coating was considered necessary in order to be sure that any spores detected in the experiments would have been newly formed and not washed from the original inoculum. Nevertheless, a subsequent experiment (expt. 4) showed that more spores could be produced from seeds treated with an aqueous suspension of spores than from alginate-coated seeds. So perhaps the use of alginate coating in most experiments actually disadvantaged the inoculant fungus.

Observation of the alginate coatings of seeds revealed that the fungus had formed a mycelial network on which spores were produced abundantly. This is similar to observations of other workers who have introduced biocontrol agents of plant pathogenes and weeds into soils or other natural environments, encased in alginate pellets (Walker & Connick, 1983; LeTakon *et al.*, 1985; Bashan, 1986;

Papavisas *et al.*, 1987;) Moreover, the alginate coating had no effect on seed germination and subsequent seedling growth. But it will be shown in Section 6 that *M. bolleyi* can undergo microcycle conidiation, whereby spores are formed directly from spores or short germ tubes. This represents an even faster means of multiplication of the fungus for potential spread down roots and it was probably prevented by the alginate casing. The dynamics of the rhizosphere as described by Bowen (1979) and in the computer model of Newman & Watson (1977) are such that an organism is advantaged by being present at a site as early as possible, before other organisms become established. For this reason and those mentioned above, it seems that direct application of spores of *M. bolleyi* to seeds would be preferable to their application in alginate gels.

Much of the work in this section involved development of appropriate methodology for studying spore production and spore movement in percolating water.

The filter technique enabled quantitative rather than qualitative estimates of the numbers of spores that could be recovered in drainage water from plant containers. It was reasonably accurate insofar as the spores of *M. bolleyi* have a characteristic, identifiable shape. But some spores evidently were misidentified (in control treatments, at least) and the method would have been much more difficult to use in normal soil because the filters would have retained slaked soil particles and spores of a range of other fungi that might have been difficult to distinguish from those of *M. bolleyi*. For example, the microconidia of *Fusarium* spp. and the spores of *Cylindrocarpon radicicola* and *Acremonium* spp. are rather similar to those of *M. bolleyi*.

Unfortunately, in many experiments there was very large variability in the spore numbers recovered on the filters from replicate containers, precluding full statistical analysis of the results and enabling only the larger treatment differences to be detected. There was evidence from some experiments that even the large amount of water used to wash the spores from such a highly porous medium as perlite was insufficient for complete and uniform collection of spores. The variability that could have been introduced into the results in this way was compounded by the variability of establishment of *M. bolleyi* on roots of individual plants (e.g. Fig.5.6) which might, in part, have been caused by interference from other microorganisms that were competitive with or antagonistic to *M. bolleyi*. An indication to this was that high numbers of spores of other fungi were occasionally observed on filters that collected drainings from inoculated and control plants.

The use of bait plants was more reliable than the filter method for detecting *M. bolleyi*. In contrast to spore counting on filters, done once per week, the bait plants were treated for 6 successive days with drainage water from individual treatment cups and the numbers of infected bait plants seemed to be good indicators of the total infectivity of the propagules rather than the numbers of spores. It is well known that spores may differ in infectivity depending on age, physiological condition and the nutrient status under which they are produced (Philips, 1965; Arora *et al.*, 1985). The high degree of infection of bait plants in appropriate treatments indicated that the spores newly produced from seed-inoculated were highly infective. For example, in experiment 1 the characteristic structures of the fungus were detected on roots of 123 out of a total 202 bait plants that received drainings from

seed-inoculated plants, and when root segments of the bait plants were plated on selective agar medium the presence of the fungus was confirmed in 186 of the total 202 plants. On the contrary only few bait plants (4 out of 205) receiving the drainage of cups with pellets+spores were seen to bear the characteristic structures of *M. bolleyi* and the fungus was present on roots of only 29 of these plants when root segments were plated on selective medium. Thus, the bait plants not only gave an estimate of the presence of *M. bolleyi* in drainage water but also gave an estimate of the relative numbers of spores (or their infectivity) insofar as the development of characteristic structures of *M. bolleyi* on roots is an indication of a high level of establishment.

It is well known that the presence of *M. bolleyi* on roots of field-grown plants is markedly under-estimated by recording the numbers and distributions of dark chlamydospore-like structures (Salt, 1976), probably because these occur only in areas of very heavy colonisation of roots. In this respect it is notable that such structures occurred mainly near the bases of roots of seed-inoculated plants (e.g. Fig. 5.4) and of bait plants. Their incidence was related to the occurrence of substantial amounts of RCD, but it is not known if this was a causal relationship or if it merely represented a coincidence of RCD and root age.

Equally important in this respect was the presence of a seed because if this was removed from seedlings on the seventh day there was a considerable reduction in the extent of chlamydospore-like structures down wheat roots (Fig. 5.4). The likely reason is that the fungus requires a high inoculum level provided by seed-derived nutrients to establish well in the rhizosphere and senescing root cortex.

A notable finding from the experiments in this section was that spores of *M. bolleyi* could be formed on colonised regions of the roots as well as on seed remains. This was demonstrated by removing the seeds from plants, when the numbers of recoverable spores were temporarily reduced but then rose again in subsequent weeks. It was shown also by incubating root pieces in moist chambers and in experiment 6 where large numbers of spores could be recovered from the older parts of roots by dilution plating. This experiment also revealed the importance of early establishment of the fungus for subsequent sporulation and, presumably, for spread down roots. For example, a delay of 11 days in introducing the fungus into tubes containing newly-sown wheat caused an approximate 10-fold reduction in numbers of spores that were produced from seeds or from the older root regions, presumably because other microorganisms had become established in the meantime.

The conditions of this study were, of necessity, artificial and they may constitute a "best case" for spread of *M. bolleyi*, on root systems. For example, fungistasis might prevent spores of *M. bolleyi* from germinating in normal soils, and microbial competition in such soils would probably moderate the ability of *M. bolleyi* to utilise seed- or root-derived nutrients and sporulate abundantly. It must nevertheless be said that all the experiments were done in unsterile conditions, so one or more microbial competitors would almost certainly have established in the spermosphere or rhizosphere, and it is generally found that fungistasis can be re-established in sterile soils by introducing any of a range of microorganisms (Lockwood, 1977). So perhaps the conditions were not so far removed from those in soil. A more serious criticism is that soils may not afford such opportunities for movement of spores in percolating water as does perlite. This is doubtless true, but it is notable

from the work of Bahme & Schroth (1987) that movement of bacteria down a soil profile in percolating water occurs to a greater extent along roots than through the bulk of soil. Movement along root surfaces would be particularly favourable for a fungus such as *M. bolleyi* which is a specialised rhizosphere inhabitant or coloniser of the root cortex of cereals. Two points seem relevant in this respect. First, the abundant sporulation of *M. bolleyi* seems ideally suited to a role of spores in colonisation of roots. Such spores are produced at an early stage in liquid culture (Section 6.4) and can be expected to be produced in water films, including those on root surfaces in normal soil. Indeed, the mycelia of this fungus in colonised root cortical tissues are ephemeral as evidenced by attempts to detect the fungus by staining of plant tissues (unpublished observation) Instead, *M. bolleyi* seems to channel its resources into either spores (presumably for dispersal in water) or into groups of chlamydospore-like structures which presumably serve in dormant survival. The second relevant point is that RCD is likely to lead to a reduced degree of contact between roots and the soil fabric, especially following a transient day-time period of high transpirational demand, when roots are known to shrink in diameter and to recover later (Scott Russell, 1977). Dead root cortical cells that are not plasmolysable would not generate turgor pressure, so the root would inevitably shrink to some degree and not recover. The spores of *M. bolleyi* are small enough to pass readily through normal filter papers (unpublished observation) and would probably be small enough to move along even narrow channels (say > 5 μm diam.) that develop around roots.

From all these considerations and the results in this section it is thus possible to construct an hypothesis. If *M. bolleyi* is applied to seeds before sowing it can exploit seed-derived nutrients

to support abundant sporulation. These seed-derived spores are an important source of inoculum for colonisation of roots in the early stages of plant growth and they can be washed down the roots in percolating water. From them the fungus establishes in the rhizosphere and exploits senescing root cortical tissues. It subsequently sporulates on the roots themselves and, because of the volume of root material, these root-derived spores subsequently become the major source of inoculum for further spread along roots. The whole sequence may be facilitated by RCD which not only provides substrates for the fungus (and a selective environment insofar as M. *bolleyi is* a weak parasite - Kirk & Deacon, 1987b) but also may serve to create narrow channels for percolating water adjacent to the roots. Rhizosphsphere biologists argue that, ideally, a microorganism should "keep pace" with the rate of root growth and spread with the growing root tip. This may or may not happen with *M. bolleyi* in natural soils, but perhaps equally important is its ability to be transported to the regions of roots where the cortex is incipiently senescent. There it can exploit the lowered host resistance and establish itself in the cortex ahead of saprophytic rhizosphere organisms. The work in this section has demonstrated the potential for such behaviour, whether or not it occurs in natural soils has yet to be determined but the hypothesis above is consistent with the common occurrence of *M. bolleyi* on cereal and grass roots in field conditions.

6. Growth and physiology of *M. bolleyi* in laboratory culture

6.1. Introduction

This section describes miscellaneous studies on growth of *M. bolleyi* in batch liquid culture and on solid media; the selection and properties of benzimidazole-tolerant mutants; sporulation as studied by videomicroscopical methods, growth of the fungus on root extracts and microscopical observations of the behaviour of *M. bolleyi* on roots. Much of this work was prompted by interest in the use of *M. bolleyi* as a seed-applied biocontrol agent of take-all or other cereal root and stem base pathogens (see Introduction). The production of mycelial biomass and of spores, and the subsequent behaviour of these, is highly relevant to commercialisation of a biocontrol agent.

Because the studies in this section were diverse, they are best introduced and discussed in the context of the experiments themselves.

6.2. Benzimidazole-tolerant mutants of *M. bolleyi*

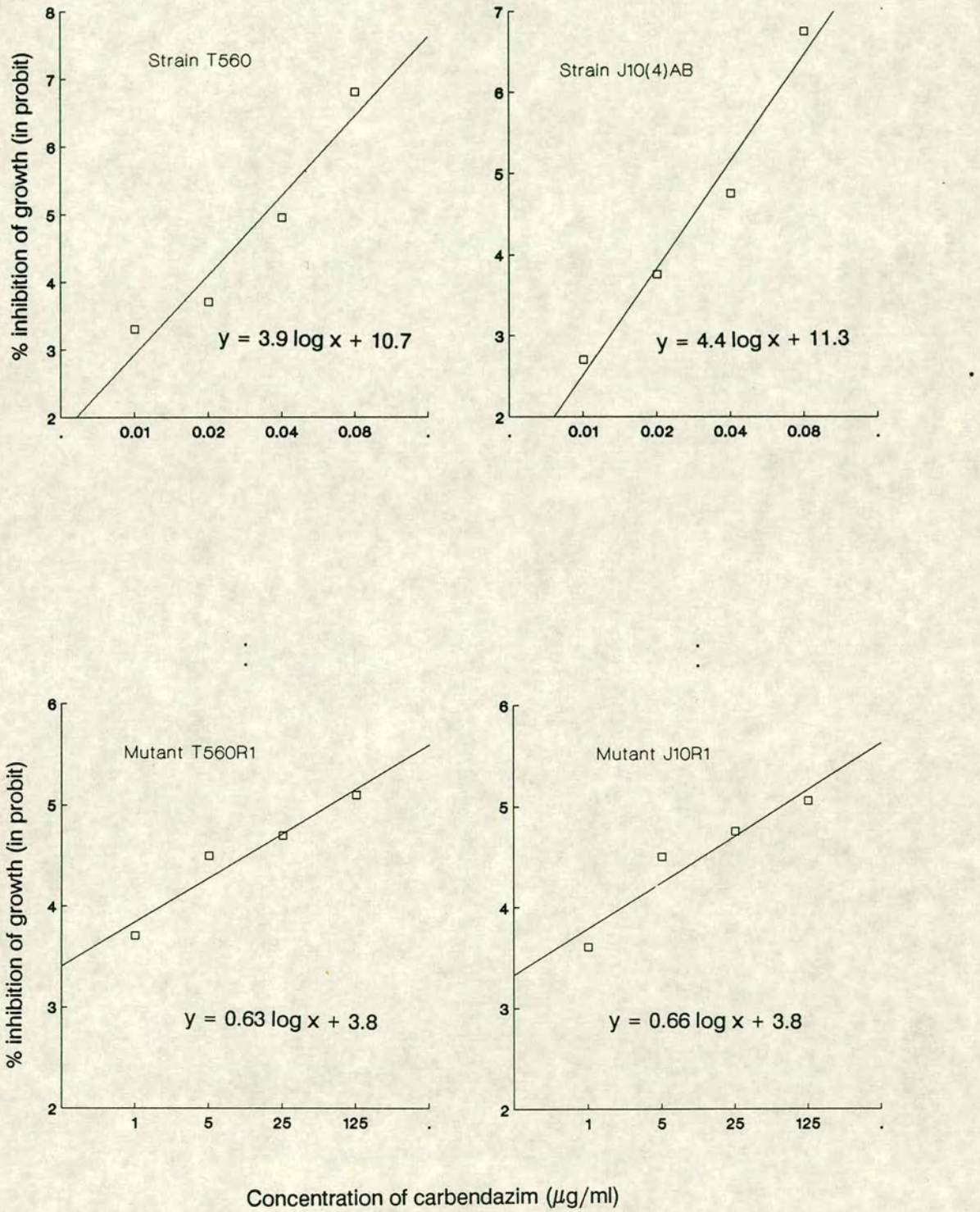
Fungicide-tolerant mutants were selected mainly for use in biocontrol studies (J.W.Deacon, unpublished) but also as a means of testing for recovery of inoculant strains in the experiments described earlier in this thesis. Their behaviour in comparison with that of wild type strains in the absence of fungicide selection is relevant to their use in ecological studies, because "marked" strains that show reduced fitness are of limited value (Andrews, 1986). In order to minimise genetic differences from wild-type strains, spontaneous mutants were selected rather than mutants generated by UV irradiation or the use of other mutagens.

Field isolates of *M. bolleyi* were grown from agar disks in 1% molasses solution, 200 ml per 500 ml Erlenmyer flask, in a rotary shaking incubator at 25°C. After 3-4 days, 10^6-10^7 spores per ml had been produced, together with considerable mycelial biomass. Carbendazim (Bavistin, Basf) was added at this stage to a final concentration of 5 μg ml^{-1}, which is sufficient completely to inhibit the growth of wild-type non resistant strains. The flasks were incubated for a further 3-4 days, then samples (0.5 ml) of the culture were spread on plates of PDA containing 5 μg ml^{-1} carbendazim. Any colonies on the plates were sub-cultured and monospore isolates were obtained from them. They were inoculated as agar disks onto PDA plates with different concentrations of carbendazim, and linear growth rates of the colonies were determined during 3 days' incubation at 25°C to determine ED_{50} values for tolerance of the fungicide.

The selection procedure was successful because several carbendazim-tolerant strains of *M. bolleyi* were obtained. As shown in Fig.6.1 for representative strains, wild-type parent isolates T560 and J10 had ED_{50} values of about 0.04 μg carbendazim ml^{-1}, based on probit plots of percent inhibition of colony extension at different levels of carbendazim in agar. The corresponding mutants, T560R1 and J10R1, had ED_{50} values of about 60 μg carbendazim ml^{-1}. Only one of the tested mutant strains (not shown) had intermediate tolerance, with an ED_{50} of 4-5 μg ml^{-1}.

The carbendazim-tolerant strains of *M. bolleyi* would be expected to be resistant to other fungicides of the benzimidazole group (Georgopoulos, 1977) but this was not tested. They were used routinely during the 3 years of this study and remained stable during routine subculture or during storage in sterile water. In colony appearance and almost all aspects of their behaviour they

Figure 6.1 Growth of wild-type strains of *M. bolleyi* (T560 and J10 and of carbendazim-tolerant mutants (T560R1 and J10R1) derived from them, in the presence of carbendazim incorporated into PDA plates.



were indistinguisable from the parent strains. However, they showed a slightly reduced growth rate compared with the parents in both liquid culture and agar culture (e.g. Table 6.12). In all these respects the mutants of *M. bolleyi* contrast markedly with carbendazim-tolerant mutants of *Trichoderma harzianum* generated by UV irradiation (Papavisas *et al.*, 1982; Papavisas & Lewis, 1983) or chemical mutagenesis (Katan *et al.*, 1984). Such mutants of *T. harzianum* are variously reported to differ from wild type in morphological characteristics, growth habit, sporulation, enhanced biocontrol capacity, cellulolytic ability and rhizosphere competence (Papavisas *et al.*, 1982; Papavisas & Liewis, 1983). Some of these features may be valuable in the generation of strains for use in biocontrol but they do not make the mutants useful as marked strains for study of the behaviour of wild-type isolates.

6.3. Growth requirements of *M. bolleyi*

A range of tests were done with liquid cultures, using 500 ml conical flasks containing 250 ml liquid culture medium to determine the growth requirments of *M. bolleyi*. The basic inorganic medium was (g per 1000 ml) as follows. $NaNO_3$, 2.0; K_2HPO_4, 1.0; $MgSO_4$ $7H_2O$, 0.5; KCl, 0.5; $FeSO_4$, 0.01. It was supplemented with microelements (mM concentrations) as follows. $MnSO_4$, 0.01; $ZnSO_4$ 0.001; $CuSO_4$, 0.001; H_3BO_3, 0.05; Na_2MoO_4, 0.0005; NaCl, 0.1; $CoCl_2$, 0.0002. In order to avoid precipitation of salts, concentrated solutions of $FeSO_4$, K_2HPO_4 and some other supplements were autoclaved separately and then added to the culture media at appropriate final concentrations. All media were supplemented with 1% glucose, except where otherwise stated. The above medium was of pH 8.2. Flasks were autoclaved for 15 min at 121 C. Strain T560 of *M. bolleyi* was used

throughout. The initial inoculum was 10^7 spores per flask, the spores being derived from agar plates or previous liquid cultures on nutritionally complete medium and being washed, centrifuged and resuspeded in sterile distilled water before use. All cultures were grown for 7 days at 25°C, on a rotary shaker (120 rpm).

At sampling (7 days) the numbers of spores per ml of culture were counted in haemocytometer. The final pH was determined. The mycelium and spores from each flask were then collected by washing the flask contents through two pre-weighed Whatman No 3 filter papers (7 cm diam), on a Buchner funnel attached to a vacuum pump. The weight of biomass was determined after drying the filters to constant weight at 80°C.

In the first set of experiments each litre of the basic mineral medium containing 1% glucose was supplemented as follows. (1) Asparagine (1.0 g).

(2) Thiamine (0.4 mg) and biotine (0.005 mg).

(3) Asparagine (1.0), thiamine (0.4 mg) and biotine 0.005 mg).

(4) Difco yeast nitrogen base (1.7 g).

(5) Difco yeast nitrogen base (1.7 g) and asparagine (1.0 g).

The Difco yeast nitrogen base contained a range of vitamins and growth factors but no amino acids or ammonium source.

Growth and spore production by the fungus is shown in Table 6.1.

It is evident from this table that *M. bolleyi* grew well using nitrate as the sole nitrogen source, provided that it was supplied with vitamins. Of the tested amendments, only the combination of thiamine and biotine was required, although higher mycelial dry weights and larger numbers of spores were produced with some of the other supplements, notably the combination of asparagine and yeast nitrogen base. This is discussed later.

Table 6.1 Growth and production of spores by *M. bolleyi* in shaken liquid culture with basic mineral salts, 1% glucose and different amendements.*

Amendement	Total oven-dry weight mg 100 ml^{-1}	Number of spores x 10^6 ml^{-1}	Final pH
Asparagine	19±2.2	0.3±0.1	6.5
Thiamine & biotine	299±19	12.3±1.6	8.3
Asparagine, thiamine & biotin	351±56	24.7±8.4	8.1
Yeast nitrogen base	306±18	27.8±2.2	8.2
Yeast nitrogene base and asparagine	351±0.8	69.7±9.8	7.7

Means ± S.E. for 3 replicates flasks after 7 days at 25°C

Table 6.2 Growth and production of spores by *M. bolleyi*, in shaken liquid culture with mineral salts, 1% glucose and different amendments.*

Amendenent	Total oven-dry weight mg 100 ml^{-1}	Number of spores x 10^6 ml^{-1}	Final pH
Biotin	23±4.3	0.01	6.7
Thiamine	314±3.5	35.7±1.7	8.6
Biotin & thiamine	302±1.6	35.0±1.5	8.7

Means ± S.E. for 3 replicates flasks after 7 days at 25°C

In a second experiment (Table 6.2) it was found that thiamine alone was needed, together with the basic mineral medium and glucose. The addition of biotin to the medium containing thiamine did not enhance growth or sporulation. This basic salts medium with thiamine and glucose was used in all further studies. In a third experiment (Table 6.3) the final biomass after 7 days' incubation was found to increase progressively with increase in glucose content of the medium, up to 3% glucose. But the number of spores produced remained constant, as did the yield coefficient, expressed as mg biomass per gram of substrate supplied.

Table 6.3 Growth and production of spores by of *M. bolleyi*, in shaken liquid culture with mineral salts and thiamine but different concentrations of glucose.

% Glucose	Total oven-dry weight mg/100 ml^{-1}	Number of spores x 10^6 ml^{-1}	Yield coefficient
1	251±7.4	36.1±2.2	0.25
2	664±2.8	36.2±2.5	0.33
3	954±8.8	30.5±3.9	0.32

Means ± S.E. for 3 replicates flasks after 7 days at 25°C

6.4. Dynamics of growth and sporulation by *M. bolleyi* in batch culture

Experiments here were designed to follow the time-course of growth and sporulation in liquid culture containing mineral salts, glucose and thiamine as described above.

Growth of filamentous fungi in liquid culture is usually determined by dry-weight determination, but this is more laborious and less precise, particularly at low concentrations of an organism, than are methods based on the light-scattering properties of cultures. Growth of unicellular microorganisms in batch culture is frequently determined by measuring turbidity (Koch, 1970). In submerged shake-flask culture, filamentous fungi can grow as dispersed mycelia (Trinci, 1972), or as dense mycelial aggregates known as pellets (Burkholder & Sinnott, 1945) or mixtures of the two.

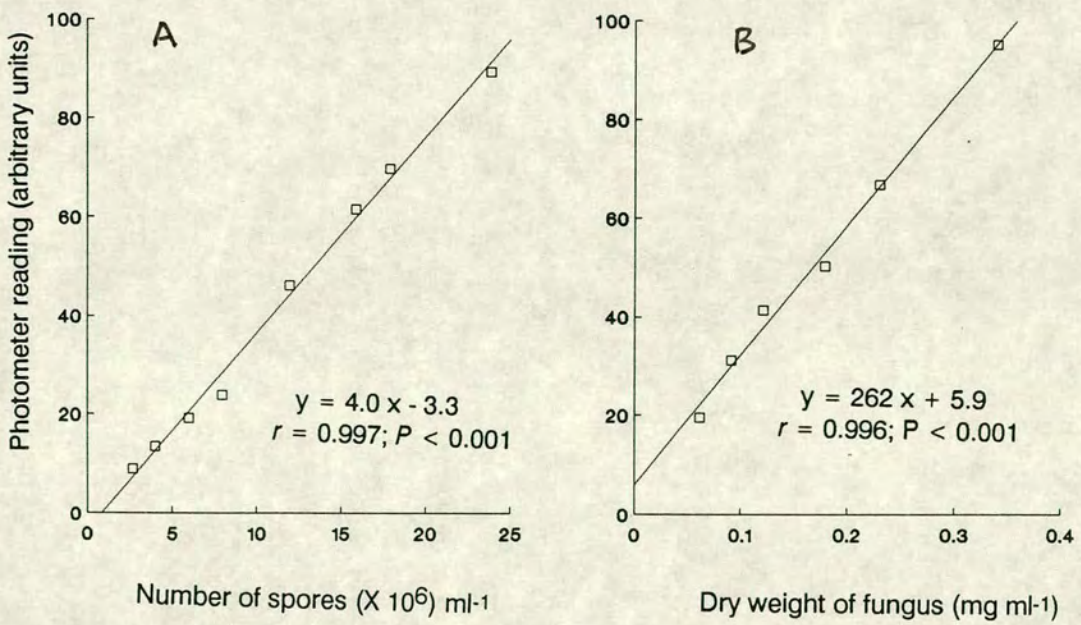
When pellets are formed there is a marked divergence from exponential growth because the fungus is not homogeneously dispersed in the medium and oxygen or substrates become growth-limiting because of problems of diffusion. Also, when a fungus grows as pellets, the turbidity of the culture cannot be used accurately to measure growth. Culture turbidity can, however, be used to assess growth of fungi as dispersed mycelia in submerged culture (Trinci, 1972; Kier *et al.*, 1976). Manipulation of the growth medium can alter fungal morphology; for example, pellet formation can be reduced by adding organic polymers such as the anionic polymer Junlon (Trinci, 1983) or the surfactant Tween 80 (Adamek, 1963; Riba & Glandard, 1980). Mycelium is then uniformly dispersed, the growth kinetics of the fungus are similar to these of unicellular microorganisms and the light scattering properties of the culture can be used as an estimate of growth.

Preliminary tests showed that the addition of sodium alginate or Junlon to culture media caused *M. bolleyi* to grow as uniformly dispersed mycelia such that turbidity of the culture could be used for measurements of growth. Junlon was particularly effective (Figure 6.2) but it posed major problems because it could not be washed from the mycelia for determination of culture weights. Sodium alginate (Sigma, high guluronic acid content) was satisfactory in reducing the number and size of pellets and was used at a concentration of 0.2% in these studies.

The fungus was grown in 250 ml Nephlos flasks with side arms, containing 100 ml of culture medium. The inoculum was 0.5 - 1 ml of spore suspension containing about 2 X 10^7 spores, which had been harvested from 3 day-old liquid cultures containing 1% glucose, washed, centrifuged and resuspended in sterile distilled water. The flasks were incubated on a rotary shaker at 200 rev. min^{-1} at 25°C. At intervals, the culture was decanted into the side arm and its turbidity was measured in an EEL nephelometer (Evans Electroselenium LTD) with a green filter and appropriate blanks. The exposed part of the flask was covered with a black cloth during the turbidity measurements. When cultures became so dense that they exceeded the limits measurement by the instrument, an appropriate dilution was made with culture medium as diluent. In all experiments the number of spores in a culture was determined at intervals by aseptically removing small samples and assessing them with a haemocytometer. Also, additional flasks were included so that the culture biomass could be determined at intervals and related to nephelometer readings. The biomass in these flasks was collected by filtration and oven-dried as described earlier. At the end of any

Figure 6.2. Morphology of *M. bolleyi* in shaken liquid culture in the presence (right) or absence (left) of Junlon (2 g l^{-1}).

Figure 6.3. Relationships between photometer readings and spore concentration (A) or total fungal dry weight (B) when *M. bolleyi* was grown for up to 72h in shaken culture in a medium containing salts, thiamine and glucose (1%).



one experiment the spores could be separated from mycelia and their weights determined separately by filtering the flask contents through Whatman lens tissue.

Figure 6.3 shows that, for cultures grown on the basic medium with thiamine and 1% glucose, there was a linear relationship between spore concentration and the photometer reading and also between culture dry weight and photometer reading when the latter was adjusted by appropriate dilution of cultures. This dilution procedure overcame the limitation that turbidity measurements underestimate biomass at high concentrations because of secondary light scattering.

Figure 6.4 shows the growth and production of spores of *M. bolleyi*, in submerged liquid culture with various concentrations of glucose. The fungus grew as dispersed mycelia, and the growth kinetics were similar to those usually obtained for unicellular microorganisms. It was possible to distinguish lag, exponential and stationary phases for glucose concentrations up to 1%. At glucose concentrations of 2 and 4%, the same general tread was seen but the growth curves had a different form, deviating from exponential growth after about 50 hours. In these latter conditions the mycelium was very dense and oxygen might have been growth-limiting.

The proportion of spores to mycelium increased progressively during growth. Most of the spores seemed to be formed during or at the end of the exponential phase. Although the total final mycelial mass was proportional to sugar concentration of the medium, the numbers of spores produced remained relatively constant at concentrations of glucose above 0.2-0.4%. This is in accordance with the findings in Table 6.3 from the previous experiment, where

Figure 6.4. Growth and production of spores by *M. bolleyi* in batch liquid cultures with 0.05-4% glucose. Each point on the curves represents the mean photometer reading (dots, left hand axis) or number of spores (*, right hand axis) for 3 replicate flasks. Curves represent lines of best fit to a logistic function.

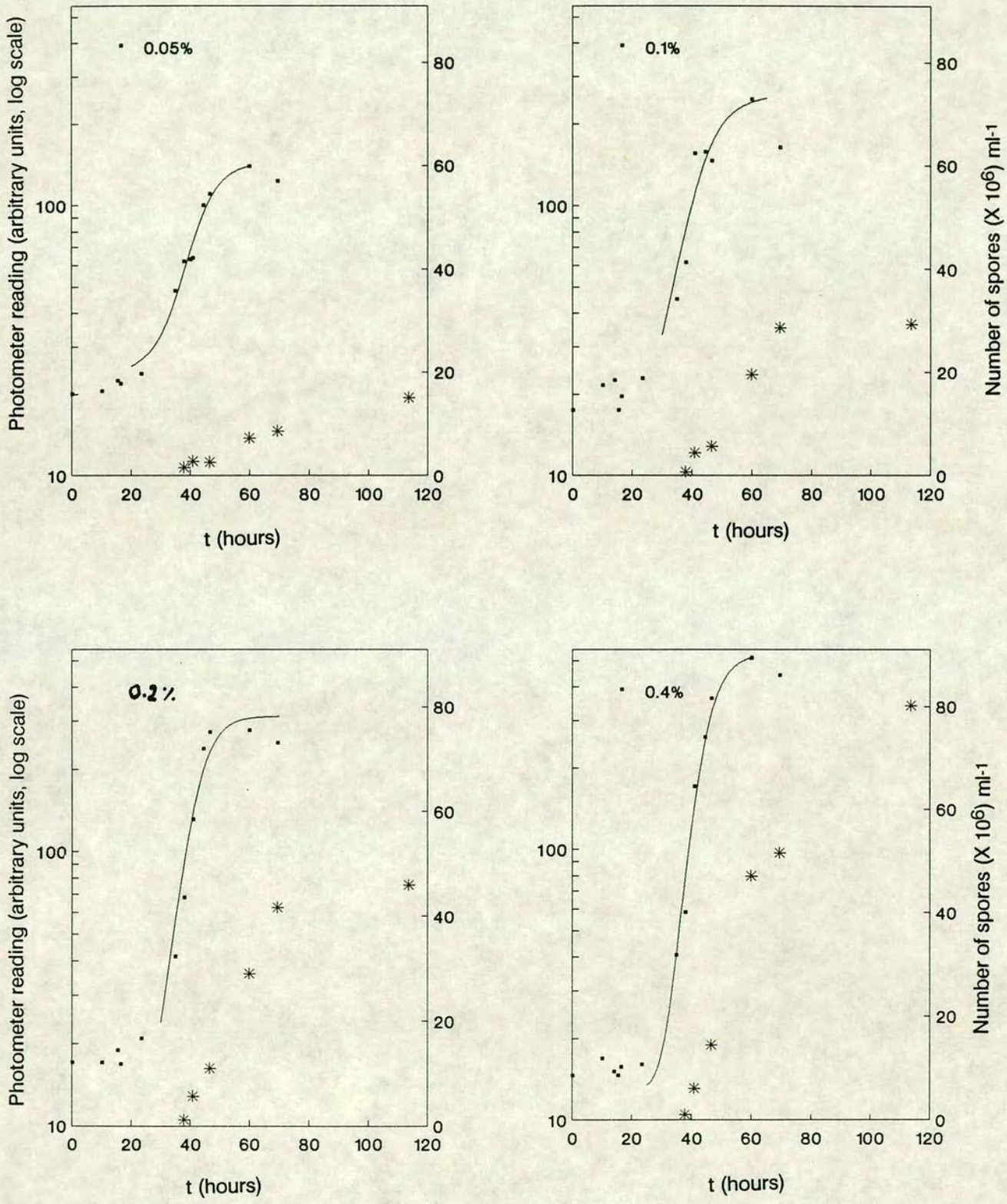
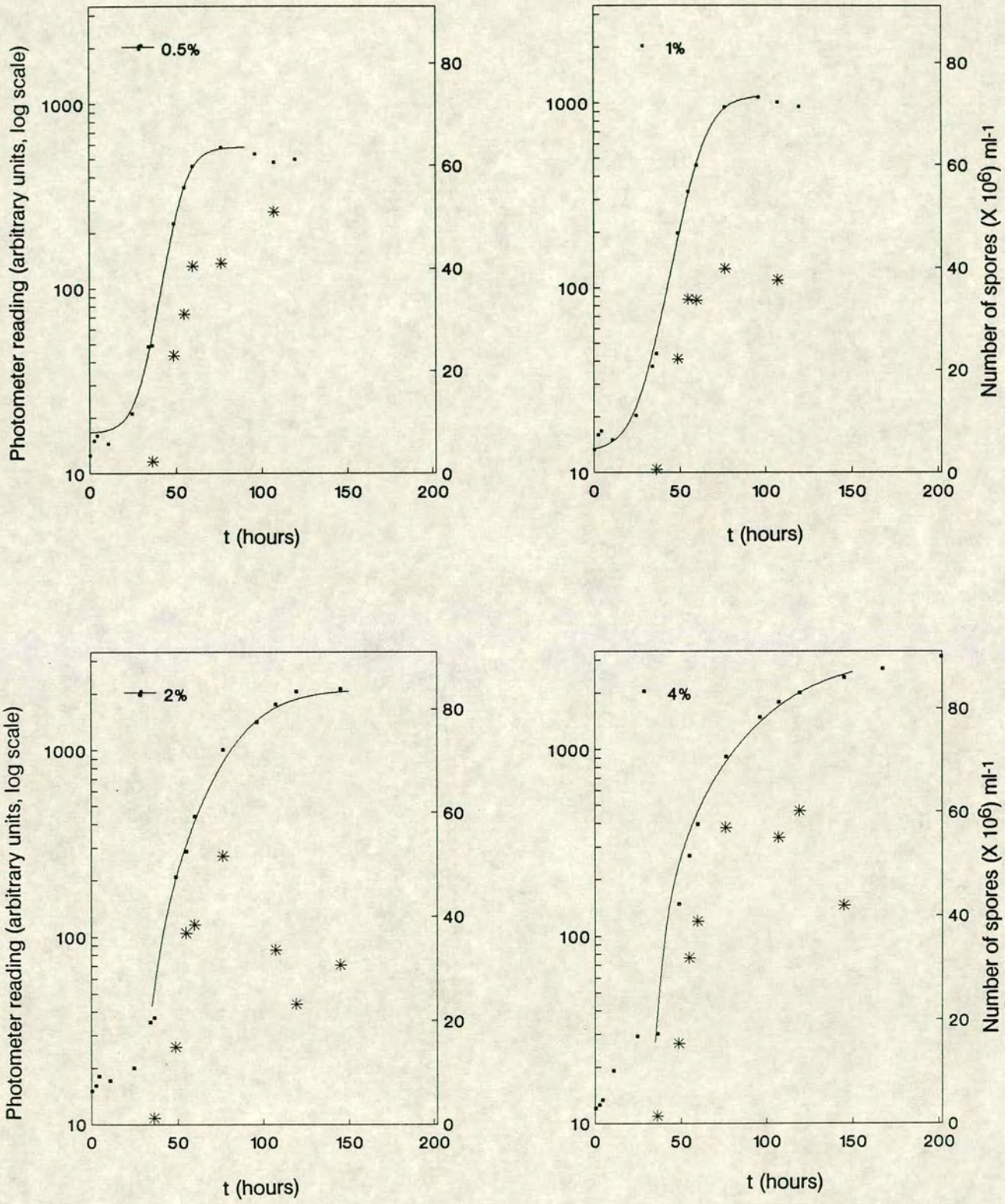


Figure 6.4. Continued.



glucose concentrations of 1, 2, and 3% gave the same numbers of spores. The mean number of spores in the 2 and 4% glucose treatments tended to decrease with extended time of incubation. A possible reason for this was that enough sugar remained in the later stages to stimulate spore germination so that the germinated spores contributed to the mycelial mass retained after passage of cultures through lens tissue. Spore germination was, indeed, seen in the cultures.

Attempts were made to estimate the contribution of spores and mycelia separately to the turbidity, by filtering the cultures through lens tissue and reassessing turbidity for the spores alone. This suggested that spores contributed more than 50% to the total light-scattering properties (measured in the nephelometer) in media containing 0.2 and 0.4% glucose, but the contribution of spores decreased at glucose concentrations higher or lower than these.

The formula of Monod (1949), $\mu = \mu_{max}\ S/K_s+S$, relates μ (the specific growth rate) to the concentration of the growth limiting nutrient (S), the maximum specific growth rate (μ_{max}) and the saturation constant (K_s) i.e. the concentration of the substrate limiting growth of the fungus when $\mu = \mu_{max}/2$.

Applying this equation, the specific growth rates (μ) in media with glucose concentrations of 0.05, 0.1, 0.2, and 0.4% were 0.050, 0.077, 0.096, and 0.110 respectively. For the same data, μ_{max} was calculated as 0.137 h^{-1} which equates to a minimum doubling time of 5.06 h; K_s was estimated as 0.085% glucose. Calculations were based only on cultures with glucose concentrations from 0.05 to 0.4% because the data points almost perfectly fitted the appropriate curve. It should be noted that the derived estimates of μ_{max} and

doubling time above relate only to the specific conditions of these experiments. *M. bolleyi* might grow faster in different conditions. It was seen earlier (Table 6.1) that many more spores were formed as a proportion of biomass when asparagine and yeast nitrogen base were used than when nitrate and thiamine were used alone in glucose-salts medium.

6.5. Studies on germination of spores of *M. bolleyi*

Microscopical observations of the liquid cultures described above revealed much variability in the size and shape of spores, which might relate to the conditions in which they are produced. When the behaviour of these spores was studied on slides bearing drops of spore suspension it was seen that they germinated either to produce a germ tube or they directly produced new spores, smaller than the parent spore, equivalent to the microcycle conidiation described for *Aspergillus niger* (Anderson & Smith, 1971) and some other fungi such as leaf-surface saprophytes (Skidmore, 1976). So a technique was used whereby the stages of spore germination and development were recorded on video tape, so that the developmental stages of individual spores could be studied.

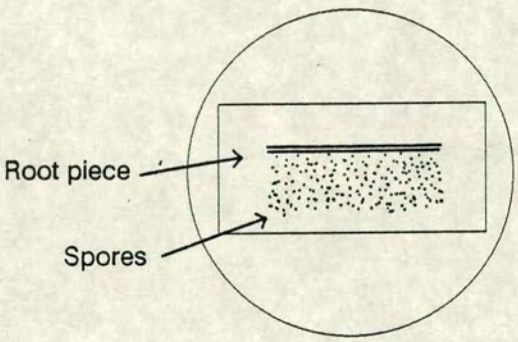
Spores harvested from 6-day-old cultures of *M. bolleyi* on PDA were suspended in sterile distilled water (SDW) and washed three times by centrifuging them at 5000 rpm for 15 min and then resuspending them in SDW. Sterile large coverslips (35 mm by 64 mm) were dipped in sterile molten agar (about 90°C) for 1-2 seconds, then were kept in a vertical position for 1-2 min and excess agar was allowed to drip off so that only a thin adhering film remained. Then each coverslip was placed on the surface of water agar in a Petri dish and the thin agar film on the coverslip was seeded with spores

of *M. bolleyi* with a sterile brush. The water in the spore suspension evaporated in these conditions so that the spores remained fixed to the agar film and their positions did not change during observation. The coverslip, seeded with spores, was removed from the agar plate and inverted onto an observation chamber (Fig.6.5) consisting of a large microscope slide with a rectangular of glass spacers 2 mm high. The inverted coverslip was sealed to the chamber with vaseline to prevent drying. The upper surface of the coverslip was cleaned and the spores were observed microscopically. A Leitz Orthoplan microscope was used with a colour video camera (Panasonic high resolution, F15 CCD) attached to it and to a video recorder (Panasonic AG-6720 S-VHS) and monitor (Panasonic BT M1420 PXG colour monitor). All observations were made with transmitted light, with X70 or X100 oil immersion objectives which gave the best image. Recordings in lower magnifications were used to map the locations of groups of spores or individual spores. The slide-chamber, when not used for observations, was kept in a larger moist chamber and incubated at 20±1 °C. At time intervals, the germination of spores was observed and recorded. Events that occurred over the period of spore germination were analysed either by comparisons of polaroid photographs, using a Mitsubishi P61-8 video copy processor attached to the video recorder, or by tracing onto an acetate overlay, calibrated with the image of a slide micrometer on the video screen.

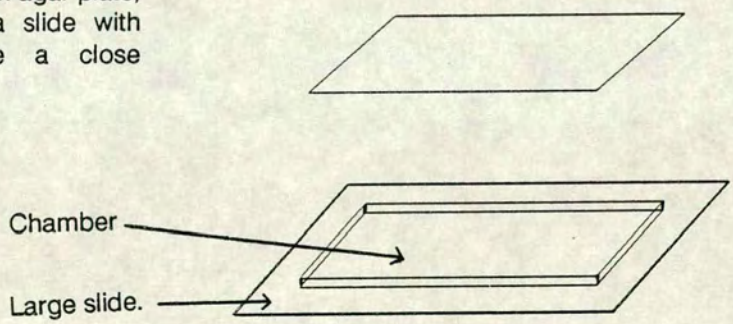
In the first such experiment the behaviour of spores was studied on water agar (2% Difco Bacto Agar) and water agar supplemented with 0.1% glucose. An "anaerobic" treatment was also included, by placing a small sterile coverslip (20x20 mm) on the surface of the agar

Figure 6.5. Schematic representation of the method used to study spore germination.

Large coverslips, seeded with spores of *M. bolleyi*, with or without a root piece; incubated on agar plate.



Coverslip removed from agar plate, inverted and sealed onto a slide with raised spacers to create a close observation chamber.



film. Observations were made 2 and 40 hours after seeding the spores. This experiment was repeated to include a treatment with 1% glucose agar, and the time-course of germination was followed more closely; also, observations were made on the position of germ-tube emergence and tropisms of germ-tubes. In a third experiment, sterile 2cm pieces of wheat or oat root were placed on the spore-seeded coverslips; germination of spores and tropism of the germ-tubes at different distances from the root pieces were studied, as well some interactions of the fungus with root hairs. In all experiments there were 3 replicate coverslips for each treatment.

6.5.1. Results

At the density of spores used in these studies (up to 1000 spores mm^{-2} of agar surface) no germination was observed beneath cover-slips placed over the agar film, except at the edge of the coverslip where oxygen diffused. In contrast "uncovered" spores (in aerated conditions) germinated within 20 h.

No gross difference of behaviour was observed between spores incubated on water agar or 0.1% glucose agar, so only data for the 0.1% medium are presented here (Table 6.4). In all cases the data are based on individual identifiable spores recorded at both 2h and 40h. At 40h, the spores could be classified into four developmental groups.

Some had not germinated and their dimensions were similar to those recorded at 2h. They may, however, have slightly increased their dimensions in the 3h since they were removed from fungal colonies and were first observed, but this was not determined. Other spores had not germinated by 40h but they had swollen markedly to about three times their original volume. The largest category

Table 6.4. Development of spores seeded on agar with 0.1% glucose and recorded by 'videomicroscopy'.

Category of behaviour	Percent of population	Dimensions (μm) after 2 h	Estimated volume (μm³) 2 h	Dimensions (μm) after 40 h	Estimated volume (μm³) 40 h
Germination by germ-tubes	45	6.4 x 2.5	26.5	8.7 x 3.5	72.8
Germination by 'budding'	15	5.7 x 2.4	21.9	7.6 x 3.0	48.2
Swollen, non-germinated	15	6.0 x 2.2	19.2	7.2 x 3.1	46.5
Non-swollen, non-germinated	25	5.6 x 2.2	14.2	5.6 x 2.2	14.2

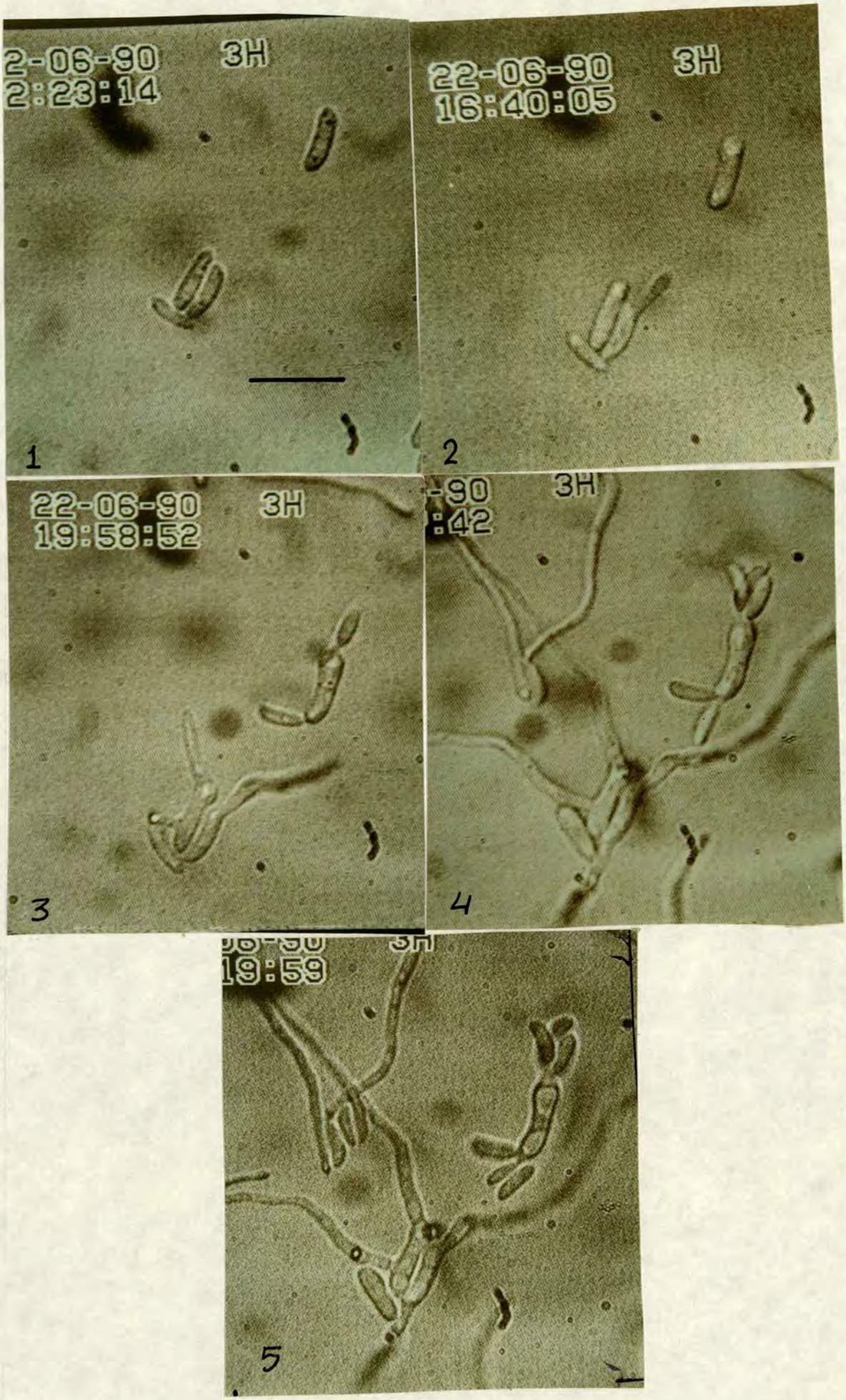
Table 6.5. Percentage of spores of *M. bolleyi* that germinated to produce germ-tubes or spores when incubated in the presence of 0, 0.1 or 1% glucose.

Glucose %	Number of spores examined	Germinated by germ-tubes (%)	Germinated by budding (%)	Non-germinated (%)
1.0	167	75	0	25
0.1	198	73	3	24
0.0	273	63	5	32

(45% of spores examined) had germinated by one or two germ-tubes, almost always from the poles of the spores; 70% of these spores had one germ-tube and 30% had two germ-tubes. Sometimes the original spore had formed a septum at its mid-point in these cases. The first-formed germ-tube was always longer than the second one, which facilitated studies of germ-tube tropism in later experiments. A smaller number of spores (15% of the total) had germinated "directly" to form further spores at one or both poles of the original spore (see Fig.6.6). The newly-formed spores were smaller than the parent because this had swelled during incubation. Some of them were of similar size to the original spores but some were smaller. None of the newly-formed spores was seen to germinate to produce further spores. In all cases of germination, whether by germ-tube or "budding" the original spore swelled considerably from its original (2h) size.

Table 6.5 shows results of a second experiment in which spores were incubated on water agar, 0.1% glucose or 1% glucose agar. Again it was found that spores germinated on water agar, and the percentage germination was not significantly increased by glucose. Spores have also been found to germinate in SDW, so it is unlikely that germination was stimulated by impurities in the water agar in this experiment. The most interesting finding was that germination by "budding" was completely suppressed in the 1% sugar medium, and this was confirmed repeatedly in other experiments. The budding sequence is shown in Fig. 6.6 for a representative spore. The first new spores were produced in 18-20 h and they continued to be produced but at a diminishing rate up to 65 h after the mother spores were seeded on water agar. Ultimately, 2-5 spores had been

Figure 6.6. Sequence of video frames showing patterns of germination of spores of *M. bolleyi* on water agar. At 17 h (2), the volume of the spores is considerably increased compared to that after 1 h (1), and one spore has already germinated. At 21 h (3), two spores have germinated to produce germ-tubes; a third spore has formed a septum and started producing new spores in both ends. At 33 h (4) and 41 h (5) more spores have been formed directly from the mother spore. The germ-tubes of other spores have elongated and grown out of the field of view, while other germ-tubes have entered the field and begun to produce new spores. Bar = 10 μm.


2-06-90
2:23:14
3H
1
22-06-90
16:40:05
3H
2
22-06-90
19:58:52
3H
3
-90
:42
3H
4
19:59
3H
5

produced from each end of the mother cell. An early stage in this sequence was the swelling of the parent cell and subsequent development of a septum. There was no evidence of germ-tube growth preceding the formation of new spores, but these seemed to be formed successively from a denticle at the pole of the parent cell. Fig.6.6 also illustrates the variable behaviour of individual spores because, in the field of view shown in the figure, only one of the four original spores germinated by budding, another had not germinated (but had swollen) by 41h, and two others had germinated to produce germ-tubes. There was no obvious difference in spore dimensions between the three spores that germinated, but only one produced other spores directly and this spore swelled more than the others and finally had a dumb-bell shape. In the later frames of the sequence shown in Figure 6.6, germ-tubes that developed from other spores outside of the field of view had grown into this field and had begun to produce spores from the germ-tubes.

The point of germ-tube emergence and subsequent orientation of germ-tube growth was studied in pairs of spores that were in contact or less than 2 μm apart, but well separated from other spores - by at least 30 μm -so they presumably were not influenced by tropic factors produced by other spores. Because of these restrictions relatively small numbers of spores associated in pairs were observed and classified in each treatment in the experiment above.

The classification of spore pairs was based on that of Jaffe (1966) as explained in Fig.6.7. There were a few situations where spore pairs could not be classified according to this system. Such pairs were ignored.

Table 6.6. Classification of spore pairs according to criteria shown in Fig. 6.7, for spores germinated on agar containing 0, 0.1 or 1% glucose.

Glucose %	No of spore pairs	*cis* ++ %	*cis* +- %	*cis* -- %	*trans* ++ %	*trans* +- %	*trans* -- %	% total spores (-)	% total spores (*cis*)
0.0	48	2	2	23	6	25	23	69***	46 NS
0.1	53	2	13	27	0	11	47	86***	42 NS
1.0	59	0	7	59	2	5	27	92***	66*

* The significance of departure from 1:1 ratio was determined from the *cis/trans* and +/- totals: *= $P < 0.05$, ***= $P < 0.001$, NS = non-significant.

Table 6.7. Germination of spores of *M. bolleyi* on water agar at different distances from wheat root pieces, after 24 h.

Distance from nearest root hair (mm)	Number of spores examined	Germinated with germ-tube (%)*	Germinated producing spores (budding) (%)	Non germinated (%)
0 (touching)	82	94 a	0	6
<1	79	92 a	0	8
1-2	67	97 a	0	3
5-6	102	83 b	7	10
20-25	187	62 c	2	36

* Figures followed by different letters are significantly different from each other ($P<0.05$), by chi-square analysis of actual numbers.

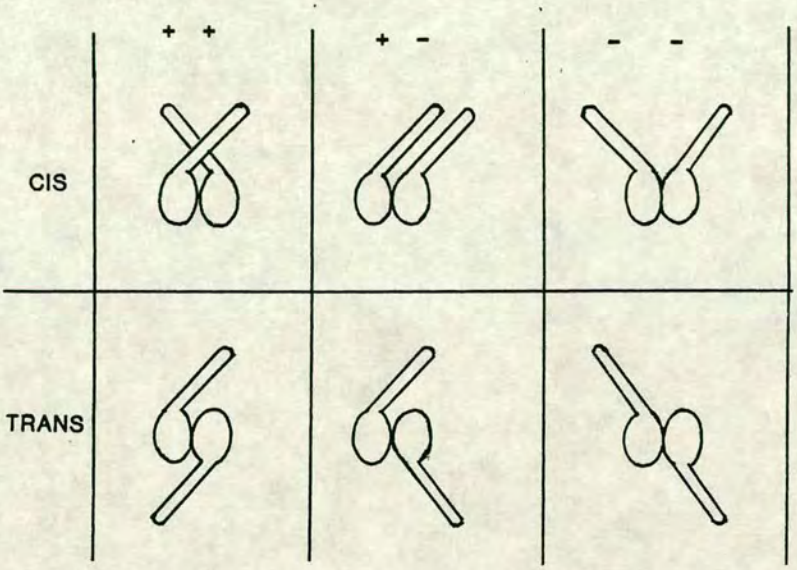


Figure 6.7. Classification of spore pairs (after Jaffe, 1966). *Cis* configuration occurs when both germ-tubes emerge from the same side of a line joining the cell centres; *trans* configuration when they emerge from opposite sides of this line. A germ-tube is scored + when the direction of germ-tube growth is towards the neighbouring spore and - when away from it.

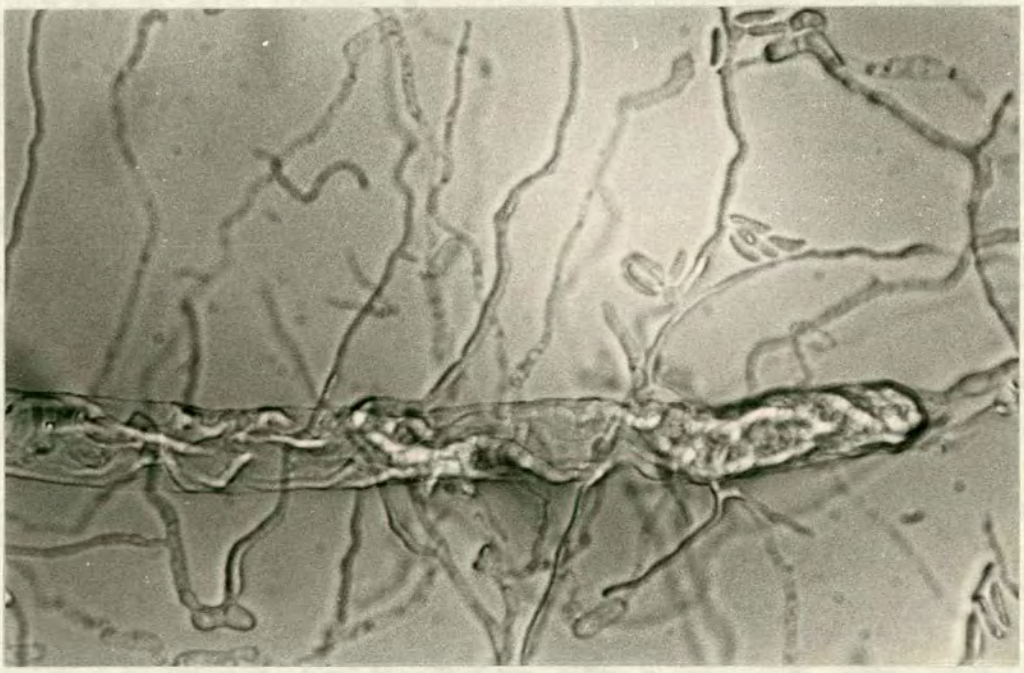


Figure 6.8. Invasion of dead wheat root hair by *M. bolleyi*.

As shown in Table 6.6 germ-tubes tended to emerge randomly in the spore pairs except in the presence of 1% glucose where there was a trend for *cis* configuration (positive autotropism, $P<0.05$). More obvious was the trend of the germ-tubes to grow away from each other (minus configuration). This was very strong in incubation media containing glucose, the values 92 and 86% being significantly different ($P<0.005$) from the 69% on water agar but even the latter was a significant departure from randomness when tested by chi-squared analysis.

Spores that germinated in the absence of glucose or with 0.1% glucose produced mycelia that formed new spores within 24 h, whereas spores that germinated in the presence of 1% glucose only produced new spores from the germ-tubes at a later stage (about 48h).

When wheat root pieces were placed on water agar, a higher percentage of spores germinated close to the root pieces than in a distance 20-25 mm from them (Table 6.7). Also, the spores close to roots always germinated to produce germ-tubes, whereas a proportion of the spores at 5-6 mm distance, or greater, from the roots germinated "directly" to form further spores. This behaviour paralleled the effects seen in glucose-free and glucose-containing agars (Table 6.5).

The presence of the root piece affected both the point of emergence of germ-tubes from spores and the subsequent orientation of germ-tubes (Table 6.8). The effect of germ-tube emergence was small but significant in some comparisons. More obvious was the fact that germ-tube growth was strongly orientated towards roots when spores were within 1 mm of the root or root hairs, 73 or 80% orientation being a significant ($P<0.001$) departure from randomness

(50%) by chi-squared analysis. In contrast, spores at 5-6 mm from the root pieces showed no tropism to roots. This distance is the same as that at which roots failed to suppress "direct" germination of spores to produce new spores (Table 6.7).

Table 6.8. Effects of wheat root pieces on point of germ-tube emergence and direction of growth of germ-tubes from spores of *M. bolleyi* on water agar, after 24 h incubation.

Distance from root (mm)	Number of germ-tubes examined	Emergence of germ-tube towards root (%)		Germ-tube growing towards root (%)	
0 (touching)	119	58	ab	73***	ac
<1	111	63**	b	80***	a
1-2	102	55	ab	61*	bcd
5-6	148	49	a	51	d

* The significance of departure from 50% (randomness) was determined by chi-squared: *= $P<0.05$, **=$P<0.01$ and ***=$P<0.001$. Figures in the same column that do not have the same letter in common are significantly different from each other ($P<0.05$) by chi-square analysis.

6.5.2. Discussion

The results of the video analysis are in some ways preliminary but reveal several points of interest about the behaviour of *M. bolleyi*. Its ability to undergo microcycle conidiation is seemingly dependent on absence of nutrients and might represent a strategy for further spread of the fungus if spores are located in moisture films in the absence of available nutrient resources. Further, both this and the ability of mycelia derived from germ-tubes to produce further spores within 24h of germination could account for spread of the fungus on roots, while the orientation of germ-tubes to root surfaces (mainly root hairs in the present study) would maximise the chances of nutrient-capture from root diffusates. Microcycle conidiation has been studied most in *Aspergillus niger* (Anderson & Smith, 1971), where it occurs when the temperature is lowered from a restrictive temperature (44°C) that prevents germ-tube outgrowth from spores but still enables the spores to swell into "giant cells". It occurs also on leaf surfaces and when spores of leaf surface saprophytes such as *Cladosporium* and *Alternaria* spp. are incubated in films of water on glass slides (Dickinson & Bottomley, 1980). Skidmore (1976) observed that *Aureobasidium pullulans* grows by yeast-like budding with a minimum of hyphal growth, and after a while a number of chlamydospores were formed. He also observed that most spores of *Chlamydosporium herbarum* germinated and grew normally but a small proportion of spores produced further conidia from short germ-tube/conidiophores within 24 h. These spores were produced on cellophane but not on agar media and were present in most instances in small numbers. It seems probable that their production is related to the nutrient status of the substrate on which the primary spore germinates. Similar

behaviour was observed by the same author in *Botrytis cinerea*, *Alternaria alternata* and *Stemphylium botryosum*. All these fungi are known as primary saprophytes on herbaceous debris (Hudson, 1971). The production of spores directly from germinating mother spores by these fungi would aid in their survival, particularly in situations where such spores germinate in the phylloplane but environmental and/or host plant conditions are unfavourable for continued growth, penetration or invasion. Fungi such as *Botrytis cinerea* are well known for the phenomenon of "latency" where colonisation is delayed until host resistance is reduced (Verhoeff 1974). The phenomenon has been reported also for many fungi such as *Neurospora crassa*, *Penicillium digitatum*, *P. italicum*, *P. urticae*, *Claviceps purpurea*, *Helminthosporium spiciferum* and *Zoophthora radicans*.

Jones & Lee (1974) observed that *Septoria tritici* also produces conidia directly from germinating spores under certain conditions and suggested that these spores could aid in the field dispersal of the pathogen. Rotem & Bashi (1969) concluded that the formation of secondary conidia in *Alternaria spp.* and *Stemphylium botryosum* was induced by various environmental factors which inhibited vegetative development from the mother conidium. *Geotrichum candidum* showed a somewhat similar situation (Park & Robinson, 1969). A similar type of microcycling probably occurs in vascular wilt fungi and has been reported for *Phialophora asteris*, the cause of aster wilt (Burge & Isaac, 1974).

Tropism of germ-tubes towards roots has been reported for several fungi, including *Pythium* and *Phytopthora* spp. (Carlile, 1983; Mitchell & Deacon, 1986) and the VAM fungus *Gigaspora gigantea* (Gemma & Koske, 1987). The factors responsible for it are unknown in most cases although volatile metabolites are implicated for *Gigaspora* and amino acids for Oomycetes such as *Pythium* spp. Other

studies on fungal tropisms include those by Robinson and his colleagues (Robinson *et al.*, 1968, Park & Robinson, 1970; Robinson, 1973) on *Geotrichum candidum*. This fungus has cylindrical spores similar to those of *M. bolleyi* and which germinate from one or both poles. For them, there is clear evidence of autotropism -the tendency of spore pairs to germinate in "cis" configurations and for germ-tubes to grow in (--) configuration. Robinson (1973) proposed that this behaviour is largely determined by oxygen gradients in water films.

Much further work seems justified to identify the responsible factors for *M. bolleyi*, but in any case the behaviour of spores observed *in vitro* in this section was found to parallel the behaviour of spores close to root pieces, indicating that the modes of spore behaviour may be ecologically relevant.

6.6. In vitro studies relating to the behaviour of *M. bolleyi* on roots

6.6.1. Video recording of growth on root hairs

In the experiments described above, germ-tubes that emerged from spores located close to root hairs were seen to exhibit characteristic patterns of behaviour. As the growing hyphae contacted dead root hairs of either wheat or oats (the death being caused by damage during handling) *M. bolleyi* often showed abundant coiling, branching and formation of appressoria. It penetrated and grew abundantly inside the dead root hairs, and in 48 hours the wheat root hairs were almost filled with mycelium (Fig.6.8). The fungus also proliferated extensively on the surface of living root hair of wheat but did not penetrate them in the first 24 h. The root hairs subsequently died, as seen by the absence of cytoplasmic streaming which was clearly visible in living root hairs. All wheat root hairs had died within 48h and most of them had then been

penetrated and extensively colonised by the fungus. There was no evidence that *M. bolleyi* hastened this death because wheat root hairs died equally rapidly in the absence of the fungus. Different behaviour was seen on root hairs of oats, which remained alive after two days' incubation and some of them even after three days - a difference that parallels that described in Section 3.12. There was little or no hyphal coiling by *M. bolleyi* around living oat root hairs but in dead oat root hairs (killed during handling) the fungus proliferated as much as in dead wheat root hairs. Attempts by the fungus to penetrate living oat root hairs were associated with the formation of conspicuous lignitubers.

6.6.2. Release of carbohydrates, amino-acids and nucleic acids from wheat root pieces.

In relation to the observed effects of root pieces on spores of *M. bolleyi* (Section 6.6.1) an attempt was made to determine the release of meterials from root pieces. This might also be relevant to the nutrients released during the normal course of RCD.

Sterile 3 cm pieces of the first-formed seminal roots of wheat, with attached tips, were produced as described in Section 2.4, were incubated in 4 ml of sterile White's mineral solution in small (5 cm diam.) Petri dishes at 25°C. The release of materials into the medium was determined after 1, 4 and 7 days incubation. There were also treatments where the root pieces were transferred to fresh solution after the first and fourth day so that exudates for the intervals 1-4 and 4-7 days were obtained. At sampling the solutions were checked for sterility by plating samples on nutrient agar, and any contaminated solutions were discarded. The root pieces at sampling were placed in 4 ml distilled water, boiled for 5 min, left overnight at 5°C and then the water was analysed for materials that had not been released from the roots. Another set of sterile wheat

root pieces were incubated on mineral agar for 1, 2, 4, 6 and 10 days, then boiled in 4 ml distilled water as above and aliquots of this were analysed. Total carbohydrates were determined by the method of Dubois *et al.* (1956). A sample (100 μl) of root "exudate" was mixed with 100 μl 5% aqueous phenol and 500 μl concentrated H_2SO_4 in a 1 ml plastic cuvette, left at room temperature for 15 min and then its absorbance at 490 nm was determined, using glucose as a standard. Total amino-acids and related compounds were assayed by the method of Moore & Stein (1954). Two reagent solutions were prepared: ninhydrin-hydridantine solution consisting of 26.6 g ninhydrin and 4 g hydridantine in 1 l cellosolve (2-ethoxyethanol), and 4N sodium-acetate buffer at pH 5.5. Root exudate (500 μl) was mixed with 375 μl of freshly-made ninhydrin-hydridantine solution and 125 μl of sodium acetate buffer in test-tubes. The tubes were capped and heated for exactly 15 min in a boiling water bath then left to cool to below 30 °C and the absorbance was read at 570 nm, using L-leucine as a standard. The nucleic acid content of the samples was estimated by ultraviolet absorption at 260 nm, using uracil as standard. All analyses were repeated three times on each sample.

Interpretation of the results (Tables 6.9-6.11) is complicated by the fact that materials could have been released from the cut ends of the root pieces as well as from death of cortical cells along the lengths of the pieces. Also, there were relatively large standard errors for some of the means. Nevertheless, the results in Table 6.9 suggest that there was little release of soluble carbohydrates or nucleic acids from root pieces during 7 days' incubation in mineral solution, compared with a substantial release of ninhydrin-positive materials (amino acids), the amounts of which accumulated with time of incubation. Comparison of Tables 6.9 and

Table 6.9. Total soluble carbohydrate, amino-acids (and related compounds) and nucleic acids released from wheat root pieces during incubation for different times in mineral nutrient solution; data per cm root length.*

Incubation period (days)	Carbohydrates (μg glucose equivalent)	Amino-acids (μg leucine equivalents)	Nucleic acids (μg uracil equivalents)
0 - 1	4.5±0.50	3.5±0.2	0.3±0.06
0 - 4	4.6±0.90	10.1±0.9	0.2±0.01
0 - 7	6.4±0.62	12.8±0.4	0.2±0.01
1 - 4	1.5±0.11	6.4±0.4	0.2±0.06
4 - 7	0.8±0.06	3.1±0.4	0.01±0.004

* Means ± S.E. for 4 replicate groups of root pieces except for 4-7 days incubation, 3 replicates.

Table 6.10. Total soluble carbohydrate, amino-acids (and related compounds) and nucleic acids extractable from root pieces <u>after</u> incubation for different times in mineral nutrient solution; data per cm root length.*

Incubation period (days)	Carbohydrates μg glucose equivalent)	Amino-acids μg leucine equivalents	Nucleic acids μg uracil equivalents
0 - 1	27.7±8.8	5.2±0.2	1.2±0.09
0 - 4	17.1±5.3	4.3±0.4	1.1±0.09
0 - 7	14.6±3.0	2.9±0.2	1.2±0.15
1 - 4	9.4±2.6	3.6±0.5	1.1±0.04
4 - 7	8.9±2.1	2.3±0.2	1.7±0.28

* Means ± S.E. for 4 replicate groups of root pieces except for 4-7 days incubation, 3 replicates.

Table 6.11. Total soluble carbohydrate, amino-acids (and related compounds) and nucleic acids extractable from root pieces after incubation for different times on mineral nutrient agar; data per cm root length.*

Incubation period (days)	Carbohydrates (μg glucose equivalent)	Amino-acids (μg leucine equivalents)	Nucleic acids (μg uracil equivalents)
1	32.0±6.3	19.7±2.1	1.6±0.1
2	17.5±3.4	12.4±1.6	1.7±0.2
4	22.8±4.4	11.7±0.4	1.6±0.1
6	24.8±6.9	8.2±0.8	1.4±0.2
10	14.7±1.3	6.3±0.3	1.9±0.2

* Means ± S.E. for 3 replicate groups of root pieces.

Table 6.12. Radial extent of growth (mm) by strains T560 and T560R1 of *M. bolleyi* after 3 days incubation at 25 °C on PDA alone or PDA supplemented with root or shoot extracts of wheat or oats.*

Medium	Strain	
	T560	T560R1
PDA	11.9±0.19	9.1±0.11
PDA + Wheat root extract	12.2±0.13	9.1±0.12
PDA + Wheat shoot extract	13.0±0.18	10.6±0.20
PDA + Oat root extract	12.0±0.15	9.9±0.08
PDA + Oat shoot extract	11.7±0.19	9.8±0.07

* Means ± S.E. of 8 replicate plates.

6.10 suggests that only a low proportion of the soluble carbohydrate or nuclei acid content of root pieces was released into solution whereas a high proportion of the total amino acid content was released. For root pieces incubated on agar it was not possible to determine the amounts of material released, but the amounts of amino acids retained in roots (Table 6.11) diminished progressively with time. From these preliminary analyses it seems that attention should be focussed particularly on the release of amino acids from detached or senescing root material in relation to the behaviour of *M. bolleyi* or other microorganisms in the rhizosphere.

6.6.3. Growth of *M. bolleyi* on oat and wheat extracts

The resistance of oats to infection by *G. graminis* var. *tritici* is often ascribed to the presence of avenacin or related saponins in oat tissues (Section 3.13). In this respect it was notable that *M. bolleyi* penetrated poorly into oat root pieces on agar media (Section 3.12) so an attempt was made to determine the sensitivity of *M. bolleyi* to toxins present in oat tissues. The methods were similar to those of Turner (1956) and Holden (1980) but involved studies in both liquid culture and agar culture.

Oat or wheat grains were sown thickly in trays of perlite in a glasshouse at 20°C. After 15 days the roots and shoots were collected separately, chopped into small pieces, and 200 g fresh weight of roots or 100 g of leaves was blended in 400 ml distilled water. The extracts were boiled to destroy plant enzymes, filtered through Whatman No 3 filter paper and stored at -24°C.

In the first experiment, Nephlos flasks were used and an attempt was made to follow the time-course of fungal growth by photometry as described in Section 6.4. *M. bolleyi* grew well in liquid media containing root or leaf extracts of either wheat or oats (data not presented). But the normal pink colour of the cultures soon was

replaced by a dark colour and the nephelometer readings became unrepresentative. The dark colour was caused by the presence of many chlamydospore-like cells, singly or in chains, and these changed the light-dispersion properties of the cultures. The effects of plant extracts were therefore studied in agar media. PDA was prepared with plant extract instead of water and both a wild type strain (T560) and its carbendazim-tolerant derivative (T560R1) were inoculated on the agar plates, from agar disks cut from colonies on PDA. After 3 days at 25°C the colony radius (8 replicate plates) of both strains was at least as great on the extract-supplement PDA as on PDA alone (Table 6.12). Indeed, in some cases the supplements slighly (and significantly) enhanced the colony extension rates. In equivalent conditions Ggt has consistently been shown to be inhibited by oat root or shoot extracts.

The notable feature of these findings is that *M. bolleyi* is insensitive to saponins or other potential inhibitors in oat tissues. So its inability to invade oat roots (e.g. living root hairs in Section 6.6.1, or senescing root pieces in Section 3.12) must be ascribed either to the maintenance of viability in the root epidermis or to the marked lignification or associated responses of oat tissues compared with those of wheat (Section 3.12).

7. Concluding discussion

Studies in this thesis showed that nuclear staining (AO) is unsuitable for plant species other than cereals. Dead tomato roots may still have stainable nuclei but their viability can be assessed with neutral red-plasmolysis. It was also shown that cytochemical methods can potentially be used for detection of early stages of cell senescence which will be valuable in more precise studies of senescence and host-parasite relations.

Tomato roots showed a different pattern of cortical senescence from that of cereals. Tomato root pieces could stay alive in agar media with even low levels of sugar, and most of the root system of glasshouse-grown plants survived long after assimilates stopped being supplied because of removal of the shoot. The fate, however, of the small feeder rootlets remains obscure and there were indications of a different type of senescence in tomato than in cereals, expressed as turn-over of the rootlets as some authors observed in other dicotyledonous plants (Wilhelm & Nelson, 1970; Huisman, 1982).

In all experiments, the cortex of cereal roots showed amounts and patterns of senescence that are well known from previous studies (Holden, 1975; Henry & Deacon 1981; Gillespie, 1986). Its rate could be altered -either accelerated or delayed- to a limited degree by external factors. Incubation of root pieces on agar media containing sucrose, IAA, $AgNO_3$ or $CoCl_2$, delayed RCD whereas media containing gibberellic acid (GA_3), or benzylaminopurine enhanced RCD, and other supplements such as gallic acid, sodium benzoate, cycloheximide or ascorbic acid had no effect. Removal of the shoot or root tip from very young seedlings resulted also in a reduced rate of RCD.

Deficiencies in nitrate or calcium increased RCD but also affected severely the growth of the whole plant. In all cases the changes in amount of RCD were small, so even though nutritional or hormonal factors can affect RCD it is unlikely any such single factor or even combination of factors are responsible for the whole phenomenon. Instead, RCD is an inherent, genetically programmed phenomenon in the Gramineae.

Several hypotheses have been proposed for the necessity and the survival advantage of the early root cortical death in cereals and grasses. The most reasonable suggestion is that this phenomenon contributes in the energy economy of the whole plant. The cortex dies and the plant economises considerably in expenditure of energy resources because it does not need to maintain these tissues (Penning de Vries, 1975), while the root expands to exploit new soil areas. It is not known yet if there is any remobilisation of nutrients from the senescing tissues towards the rest of the plant. It is known, however, that this happens extensively in other senescing plant tissues and organs such as senescing leaves. The way the cortical cells senesce and die seems to be very well ordered and not simply a cell collapse. The loss of nuclei in these tissues and absence of any stainable (with AO) material suggests a breakdown of the double stands of DNA at some phase of the process. This loss of DNA is consistent with the hypothesis of a remobilisation of nutrients. This recently was suggested by Robinson (1990), in the different context of remobilisation of phosphorus. It is obvious that further study is needed in this area in terms of carbon balance in the root and the plant as a whole and in relation to the amount and nature of nutrients released into the rhizosphere during RCD.

The attempt to use root pieces or manipulate whole plants in order to change the rate of RCD and so study the invasion of the cortices by fungi was not too successful because the cortices of cereal root pieces died quickly in all conditions and the treatments used for the whole plants had severe effects on other aspects of plant physiology.

Studies on tomato and *P. lycopersici* gave no clear evidence of a direct role of root senescence in infection. Instead, the higher incidence of infection in old compared with young root regions might be explained in terms of inoculum build-up by *P. lycopersici* in the older, denser rooting zones facilitated perhaps by 'shedding' of terminal rootlets or root branches (Wilhelm & Nelson 1970). Anyhow, *P.lycopersici* was more aggressive than *M. bolleyi* on root pieces of either wheat or tomato, consistent with the yield reduction known to be associated with this fungus (Last & Ebben, 1966) and the absence of reliable evidence of yield reductions caused by *M. bolleyi*. The failure to find a clear pattern of senescence-related invasion in tomato and the difficulty of studying tomato root systems led to concentration on the cereal- *M. bolleyi* association. There was little value in continuing comparative studies of these host-parasite systems.

M. bolleyi showed in many instances an increased ability to invade senescing root cortices. Many results in this thesis support the view that *M. bolleyi* is a weak parasite and that it exploits natural or artificially-induced senescence rather than being a major determinant of senescence. In root pieces with declining resistance it could, however, enhance the rate of RCD. This might explain, for example, why it is associated with death of flax in field conditions

where standing water would have caused serious debilitation of the plants (Black & Brown, 1986). It is also known that *M. bolleyi* can damage root tips from overwhelming inoculum levels (Fitt & Hornby, 1978). However, all these reports relate to extreme conditions and it may be noted that even characteristically non-parasitic fungi can cause damage to plants in such extreme conditions.

Several new aspects of the biology of *M. bolleyi* were revealed in this thesis, and together they suggest an hypothesis to account for the behaviour of this fungus on roots. It was found, for example, that *M. bolleyi* exploits seed materials for production of spores which can be spread down roots in perlite. The fungus can thus become established in the roots and sporulate there (at least in the conditions of this work), producing further inoculum for colonisation of roots. This feature is different from the behaviour of *G. graminis* and *P. graminicola*, which probably do not sporulate on roots or the spores of which are ineffective for establishment of infection. In all experimental work, the extent of these fungi down roots from an inoculum can be traced to individual hyphae that grow from the initial focus of infection. Unfortunately the hyphae of *M. bolleyi* are hyaline and thus cannot be traced along roots. In any case, they are ephemeral: the fungus seems to convert its resources either to spores or to groups of dark chlamydospore-like cells after a limited phase of mycelial growth. The production of spores directly from other spores in the absence of nutrients (glucose or root diffusates) is of interest and possible relevance for spread through soil or along roots. The effect of high sugar levels in delaying sporulation from germ-tubes also is relevant in this respect. But the analysis of nutrients released from root pieces,

although preliminary, suggest that more attention might be focused on amino acids than on sugars in relation to the behaviour of *M. bolleyi* in the rhizosphere. Of interest in this respect was the finding that the growth requirements of *M. bolleyi* are relatively simple. The fungus needs only inorganic nitrogen and other salts, thiamine and glucose. However, sporulation was greatly enhanced in culture media containing vitamins in yeast nitrogen base, alone or in combination with asparagine.

Further work on the behaviour of *M. bolleyi* in the rhizosphere seems justified, especially in relation to the potential of this fungus to act as a biocontrol agent of take-all and other cereal pathogens (see Introduction) and to its use as a seed-applied inoculant in field conditions. It is suggested that *M. bolleyi* might be dispersed in percolating water in soil, in which case it could logically be expected to travel further down roots than in the bulk of soil because the path along roots is more direct than in the bulk of soil (Bahme & Schroth, 1987). The production of small "secondary" spores could facilitate this spread, as could any contraction of the root cortex in temporary conditions of plant water deficit (Scott Russel, 1977). The latter idea is particularly attractive (although unproven) because senescent or senescing cortical cell layers would not be expected to re-expand to the same degree as do living cortical layers that can generate turgor pressure. Indeed, Jupp & Newman (1987) found that drying of grass root cortices can lead to cell death. A fungus such as *M. bolleyi* that exploits incipiently senescing root cells could thus, in percolating water, be carried down roots to near the maximum extent of cortical

senescence and be placed such that it could exploit these cells as they senesce.

In further work it should be possible to test this hypothesis or, at least, components of it. Studies on spore movement in soils would probably best be done using cores of natural soils with stable structure rather than disturbed soils because the former should be more resistant to slaking of clay particles by water passed through them. It should also be possible to assess the degree of contraction and expansion of cereal roots in simulated daily cycles of transpirational demand, using either borescopes (Rush *et al.*, 1984) or video techniques like those explored here and which provide hitherto unrecorded details of events in the root zone. All such soil studies are facilitated by the availability of mutants of *M. bolleyi* (Section 6.2) that behave essentially similarly to wild-type strains and thus are potentially useful for assessing the behaviour of *M. bolleyi* added as inoculum, even to soils that already contain populations of this common fungus.

Summary of analysis of variance of experiment in Section 3.10, involving preincubation time (P), day of sampling (D) and treatment (T). Data (numbers of stainable nuclei) were transformed in log (X+1). Each cortical layer was analysed separately. Significant items are indicated by: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; NS, non-significant.

		Root cell layer						
	DF	1st	2nd	3rd	4th	5th	6th	Endodermis
Pre-incubation (P)	2	***	***	***	***	***	*	NS
Sampling day (D)	4	***	***	***	***	**	**	*
Treatment (T)	2	**	***	***	***	***	***	***
P x D	8	***	*	*	*	NS	*	NS
P x T	4	*	***	***	***	NS	NS	NS
D x T	8	NS	NS	NS	NS	NS	NS	NS
Residual	16							

Pre-incubation x Treatment
Log (nuclei+1)

LAYER 1

Pre-incubation in days	Treatment MB	PDA	WA
0	0.067	0.245	0.223
2	0.000	0.003	0.053
4	0.000	0.000	0.016

S.E.D. 0.033
L.S.D.(1%) 0.096

LAYER 2

Pre-incubation in days	Treatment MB	PDA	WA
0	0.073	0.582	0.446
2	0.057	0.172	0.154
4	0.008	0.008	0.045

S.E.D. 0.058
L.S.D.(1%) 0.169

LAYER 3

Pre-incubation in days	Treatment MB	PDA	WA
0	0.126	0.671	0.520
2	0.144	0.331	0.267
4	0.046	0.031	0.097

S.E.D 0.041
L.S.D.(1%) 0.120

LAYER 4

Pre-incubation in days	Treatment MB	PDA	WA
0	0.209	0.658	0.467
2	0.155	0.372	0.274
4	0.041	0.079	0.114

S.E.D. 0.032
L.S.D.(1%) 0.093

LAYER 5

Pre-incubation in days	Treatment MB	PDA	WA
0	0.253	0.638	0.615
2	0.261	0.447	0.474
4	0.072	0.160	0.241

S.E.D 0.077
L.S.D.(1%) 0.225

LAYER 6

Pre-incubation in days	Treatment MB	PDA	WA
0	0.509	0.625	0.756
2	0.551	0.648	0.754
4	0.271	0.596	0.615

S.E.D. 0.076
L.S.D.(1%) 0.222

LAYER 7 (Endodermis)

Pre-incubation in days	Treatment MB	PDA	WA
0	0.353	0.438	0.785
2	0.387	0.437	0.668
4	0.320	0.466	0.526

S.E.D. 0.094
L.S.D.(1%) 0.274

Summary of analysis of variance of experiment in Section 3.11 involving wheat roots attached or detached from seedling (R), day of sampling (D) and treatment i.e. two types of inoculum of *M. bolleyi* and control (T). Numbers of stainable nuclei in each cell layer of 50 μm sections were transformed to log (X+1). Each cortical layer was analysed separately. Significant items are indicated by: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; NS, non-significant.

		Cell layer						
	DF	1	2	3	4	5	6	7
Root attached or detached (R)	1	NS	***	***	***	*	*	NS
Day of sampling (D)	5	***	***	***	***	*	NS	*
Treatment (T)	2	NS	***	NS	NS	NS	NS	NS
R x D	5	NS	**	*	**	NS	NS	NS
R x T	2	NS	*	NS	NS	NS	NS	NS
D x T	10	NS	NS	NS	NS	NS	NS	NS
Residual	10							

Root excision x Treatment

Treatment	Layer 1		Layer 2		Layer 3	
	Att.	Det.	Att.	Det.	Att.	Det.6
Control	0.286	0.254	0.567	0.509	0.566	0.509
Mb-PDA	0.217	0.083	0.458	0.246	0.599	0.370
Mb-spores	0.292	0.232	0.599	0.405	0.625	0.386
S.E.D.	0.059		S.E.D.	0.037	S.E.D.	0.056
L.S.D.(1%)	0.187		L.S.D.(1%)	0.117	L.S.D.(1%)	0.177

Treatment	Layer 4		Layer 5		Layer 6	
	Att.	Det.	Att.	Det.	Att.	Det.6
Control	0.552	0.459	0.581	0.564	0.743	0.759
Mb-PDA	0.533	0.335	0.547	0.411	0.722	0.545
Mb-spores	0.473	0.400	0.580	0.422	0.746	0.549
S.E.D.	0.037		S.E.D.	0.060	S.E.D.	0.068
L.S.D.(1%)	0.117		L.S.D.(1%)	0.190	L.S.D.(1%)	0.215

Treatment	Layer 7 (Endodermis)	
	Att.	Det.
Control	0.743	0.759
Mb-PDA	0.722	0.545
Mb-spores	0.746	0.549
S.E.D.	0.068	
L.S.D.(1%)	0.215	

Summary of analysis of variance of experiment in Section 3.11, involving wheat roots attached or detached from seedling (R), day of sampling (D) and treatment i.e. two types of inoculum of *P. lycopersici* and control (T). Numbers of stainable nuclei in each cell layer of 50 μm sections were transformed to log (X+1). Each cortical layer was analysed separately. Significant items are indicated by: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; NS, non-significant.

		Cell layer						
	DF	1	2	3	4	5	6	7
Root attached or detached (R)	1	*	*	*	NS	NS	NS	NS
Day of sampling (D)	4	***	***	***	*	NS	NS	NS
Treatment (T)	2	NS	**	**	NS	NS	NS	NS
R x D	4	NS	NS	NS	NS	NS	NS	NS
R x T	2	NS	NS	*	NS	NS	NS	NS
D X T	8	NS	NS	*	NS	NS	NS	NS
Residual	8	NS						

Root excision X Treatment

Treatment	Layer 1		Layer 2		Layer 3	
	Att.	Det.	Att.	Det.	Att.	Det.6
Control	0.200	0.121	0.578	0.410	0.655	0.574
Pl-PDA	0.159	0.051	0.436	0.240	0.564	0.326
Pl-spores	0.296	0.179	0.616	0.553	0.666	0.726
S.E.D.	0.059		0.049		0.054	
L.S.D.(1%)	0.198		0.218		0.163	

Treatment	Layer 4		Layer 5		Layer 6	
	Att.	Det.	Att.	Det.	Att.	Det.
Control	0.566	0.533	0.603	0.585	0.796	0.796
Pl-PDA	0.588	0.416	0.576	0.509	0.788	0.728
Pl-spores	0.551	0.608	0.602	0.579	0.772	0.770
S.E.D.	0.057		0.049		0.053	
L.S.D.(1%)	0.119		0.163		0.179	

Treatment	Layer 7 (endodermis)	
	Att.	Det.
Control	0.755	0.748
Pl-PDA	0.722	0.678
Pl-spores	0.736	0.733
S.E.D.	0.028	
L.S.D.(1%)	0.094	

Summary of analysis of variance of experiment in Section 3.12, involving wheat roots attached or detached from seedling (R), day of sampling (D) and treatment (T) i.e. *M. bolleyi* inoculum, two types of *G. graminis* var *tritici* inoculum and control. Numbers of stainable nuclei in each cell layer of 50 μm sections were transformed to log (X+1). Each cortical layer was analysed separately. Significant items are indicated by: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; NS, non-significant.

		Cell layer						
	DF	1	2	3	4	5	6	7
Root (R)	1	NS	*	NS	**	NS	*	*
Day (D)	3	**	***	***	***	**	***	**
Treatment (T)	3	*	**	***	***	***	***	***
RXD	3	NS	*	NS	NS	NS	NS	NS
RXT	3	NS	NS	NS	NS	NS	*	NS
DXT	9	NS	NS	*	NS	NS	NS	NS
Residual	9							

Root X Treatment

	Layer 1		Layer 2		Layer 3	
	Att.	Det.	Att.	Det.	Att.	Det.6
Control	0.199	0.179	0.392	0.324	0.476	0.503
Mb	0.168	0.010	0.315	0.081	0.511	0.227
Ggt-WA	0.044	0.000	0.153	0.020	0.166	0.095
Ggt-PDA	0.000	0.000	0.010	0.000	0.028	0.020
S.E.D.	0.0660		0.0912		0.0757	
L.S.D.(1%)	0.215		0.246		0.235	

	Layer 4		Layer 5		Layer 6	
	Att.	Det.	Att.	Det.	Att.	Det.
Control	0.463	0.430	0.498	0.553	0.824	0.823
Mb	0.560	0.243	0.650	0.304	0.825	0.345
Ggt-WA	0.179	0.075	0.146	0.126	0.224	0.177
Ggt-PDA	0.058	0.037	0.112	0.064	0.115	0.099
S.E.D.	0.0722		0.1072		0.1049	
L.S.D.(1%)	0.235		0.348		0.341	

	Layer 7 (endodermis)	
	Att.	Det.
Control	0.816	0.792
Mb	0.740	0.337
Ggt-WA	0.337	0.202
Ggt-PDA	0.284	0.161
S.E.D.	0.1342	
L.S.D.(1%)	0.436	

Summary of analysis of variance of experiment in section 3.12, involving barley roots attached or detached from seedling (R), day of sampling (D) and treatment (T) i.e. *M. bolleyi* inoculum, two types of *G. graminis* var *tritici* inoculum and control. Numbers of stainable nuclei in each cell layer of 50 μm sections were transformed to log (X+1). Each cortical layer was analysed separately. Significant items are indicated by: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; NS, non-significant.

		Cell layer						
	DF	1	2	3	4	5	6	7
Root (R)	1	*	***	**	***	***	***	**
Day (D)	3	***	***	**	**	*	**	**
Treatment (T)	3	**	***	***	***	***	***	***
RXD	3	*	NS	NS	NS	NS	NS	NS
RXT	3	NS	NS	*	NS	NS	*	NS
DXT	9	NS	NS	NS	NS	NS	NS	NS
Residual	9							

Root x Treatment

	Layer 1		Layer 2		Layer 3	
	Att.	Det.	Att.	Det.	Att.	Det.
Control	0.264	0.267	0.723	0.553	0.652	0.611
Mb	0.188	0.000	0.523	0.112	0.702	0.168
Ggt-WA	0.086	0.020	0.254	0.058	0.212	0.058
Ggt-PDA	0.051	0.000	0.170	0.000	0.169	0.000
S.E.D.	0.0623		0.0905		0.1059	
L.S.D.(1%)	0.202		0.294		0.344	

	Layer 4		Layer 5		Layer 6	
	Att.	Det.	Att.	Det.	Att.	Det.
Control	0.693	0.512	0.750	0.509	0.884	0.763
Mb	0.748	0.177	0.753	0.148	0.818	0.220
Ggt-WA	0.200	0.081	0.209	0.058	0.235	0.119
Ggt-PDA	0.159	0.000	0.199	0.010	0.250	0.010
S.E.D .	0.1075		0.1253		0.1153	
L.S.D.(1 %)	0.349		0.407		0.375	

	Layer 7 (endodermis)	
	Att.	Det.
Control	0.840	0.775
Mb	0.734	0.219
Ggt-WA	0.366	0.173
Ggt-PDA	0.356	0.064
S.E.D.	0.1211	
L.S.D.(1%)	0.394	

Summary of analysis of variance of experiment in section 3.12, involving rye roots attached or detached from seedling (R), day of sampling (D) and treatment (T) i.e. *M. bolleyi* inoculum, two types of *G. graminis* var *tritici* inoculum and control. Numbers of stainable nuclei in each cell layer of 50 μm sections were transformed to log (X+1). Each cortical layer was analysed separately. Significant items are indicated by: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; NS non-significant.

Source of variation	DF	Cell layer 1	2	3	4	5	6
Root (R)	1	NS	*	**	***	***	***
Day (D)	3	***	***	**	***	**	**
Treatment (T)	3	*	***	***	***	***	**
RXD	3	NS	NS	NS	*	NS	NS
RXT	3	*	**	**	*	*	NS
DXT	9	*	NS	NS	NS	NS	NS
Residual	9						

Root X Treatment

	Layer 1 Att.	Det.	Layer 2 Att.	Det.	Layer 3 Att.	Det.
Control	0.095	0.234	0.425	0.555	0.539	0.678
Mb	0.149	0.000	0.469	0.064	0.629	0.124
Ggt-WA	0.249	0.171	0.353	0.229	0.479	0.243
Ggt-PDA	0.096	0.010	0.144	0.051	0.189	0.119
S.E.D.	0.063		0.077		0.099	
L.S.D.(1%)	0.203		0.250		0.322	

	Layer 4 Att.	Det.	Layer 5 Att.	Det.	Layer 6 Att.	Det.
Control	0.725	0.648	0.883	0.820	0.901	0.751
Mb	0.642	0.209	0.866	0.246	0.826	0.212
Ggt-WA	0.496	0.230	0.587	0.323	0.620	0.321
Ggt-PDA	0.206	0.126	0.286	0.168	0.367	0.151
S.E.D.	0.086		0.117		0.139	
L.S.D.(1%)	0.280		0.380		0.453	

Summary of analysis of variance of experiment in Section 3.12, involving oat roots attached or detached from seedling (R), day of sampling (D) and treatment (T) i.e. *M. bolleyi* inoculum, two types of *G. graminis* var *tritici* inoculum and control. Numbers of stainable nuclei in each cell layer of 50 μm sections were transformed to log (X+1). Each cortical layer was analysed separately. Significant items are indicated by: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; NS, non-significant.

Source of variation	DF	Cell layer 1	2	3	4	5	6	7
Root (R)	1	*	***	***	**	**	***	***
Day (D)	3	**	***	***	***	***	***	***
Treatment (T)	3	*	NS	NS	NS	NS	NS	*
RXD	3	**1	*1	*1	NS	NS	*1	NS
RXT	3	NS	NS	NS	NS	NS	NS	NS
DXT	9	NS	NS	NS	NS	NS	NS	NS
Residual	9							

Root X Treatment

	Layer 1 Att.	Det.	Layer 2 Att.	Det.	Layer 3 Att.	Det.6
Control	0.687	0.401	0.846	0.495	0.597	0.396
Mb	0.460	0.189	0.706	0.237	0.704	0.213
Ggt-WA	0.393	0.292	0.678	0.331	0.520	0.242
Ggt-PDA	0.317	0.238	0.567	0.330	0.489	0.290
S.E.D.	0.117		0.130		0.088	
L.S.D.(1%)	0.380		0.424		0.286	

	Layer 4 Att.	Det.	Layer 5 Att.	Det.	Layer 6 Att.	Det.6
Control	0.480	0.317	0.426	0.310	0.745	0.404
Mb	0.526	0.156	0.439	0.126	0.731	0.211
Ggt-WA	0.373	0.200	0.379	0.211	0.470	0.270
Ggt-PDA	0.332	0.225	0.321	0.219	0.654	0.287
S.E.D.	0.100		0.094		0.115	
L.S.D.(1%)	0.325		0.305		0.373	

	Layer 7 (endodermis) Att.	Det.
Control	0.721	0.428
Mb	0.591	0.195
Ggt-WA	0.533	0.285
Ggt-PDA	0.410	0.292
S.E.D.	0.087	
L.S.D.(1%)	0.284	

Summary of the analysis of variance of experiment in Section 3.6, involving day of sampling (D), medium at base and medium at apex of the root piece. Wheat root pieces incubated in split plates; numbers of nucleate cortical cell layers analysed separately for each root region. Significant items are indicated by * $P<0.05$; ** $P<0.01$ and *** $P<0.001$; NS, non-significant.

ROOT TIPS ATTACHED

		Root region			
	DF	A(base)	B	C	D(tip)
Day of sampling (D)	1	NS	NS	**(a)	**(a)
Medium at base (B)	2	NS	**(b)	NS	NS
Medium at tip (T)	2	*(c)	**(c)	*(c)	*(b)
D x B	2	NS	NS	NS	NS
D x A	2	NS	NS	**	NS
B x A	4	NS	NS	*(d)	NS
D x B x A	4	NS	NS	NS	NS
Residual	68 (4 missing values)				

ROOT TIPS REMOVED

		Root region			
	DF	A(base)	B	C	D(tip)
Day of sampling (D)	1	NS	***(a)	***(a)	**(a)
Medium at base (B)	2	NS	*(b)	**(b)	**(b)
Medium at tip (T)	2	NS	NS	NS	NS
DXB	2	NS	NS	NS	NS
DXA	2	NS	NS	NS	NS
BXA	4	NS	**(d)	***(d)	*(d)
DXBXA	4	NS	NS	NS	NS
Residual	72				

(a) Numbers of nuclei decrease with time.
(b) Decreased numbers of nuclei in WA compared with SMA and MA.
(c) Decreased numbers of nuclei in WA and MA compared with WSA.
(d) WSA has non-additive effect when it is at both ends of root piece.

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